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SINGER MICROMANIPULATORS

[4]

[5]

The Journal of
General Microbiology

Editors: B. C. J. G. KNIGHT and A. F. B. STANDFAST

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The Interference Microscope as a Refractometer for Liquids

By S. IVERSEN and F. H. SMITH
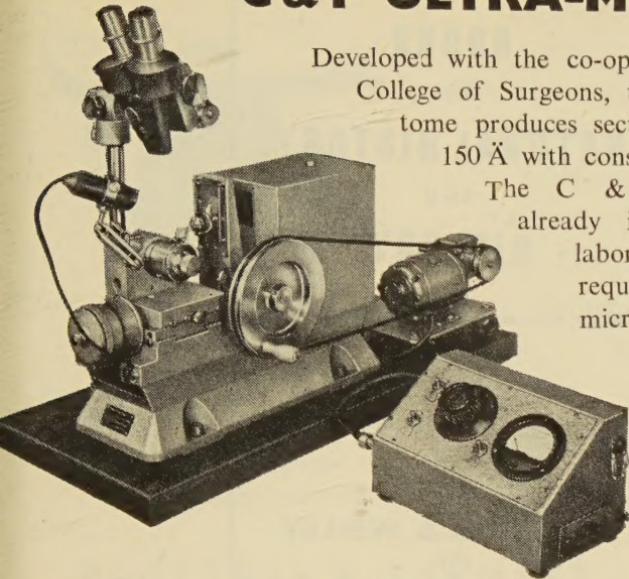
From the Department of Anatomy, Royal College of Surgeons of England, London, W.C. 2, and Research Laboratories of Messrs. Charles Baker, Croydon)

With one plate (fig. 2)

SUMMARY

Determination of the refractive index of liquids by the interference microscope was made possible by the use of a specially constructed holder for the liquid. This ensures a permanent lateral separation between the reference and the object areas and, as the depth of the object area is continuously increasing, a system of fringes is observed. The spacing of the fringes is a function of the refractive index of the liquid. By determining this spacing, a measurement of the refractive index is obtained. An accuracy of at least 0·001 in refractive index is obtainable by a single measurement.

THE interference microscope makes it possible to measure the optical path difference, and thereby the refractive index, of two laterally separated features. In this paper its application as a refractometer for liquids is described.

In order to obtain a permanent lateral separation between the liquid and the reference area, a special liquid holder was designed. This consists of two semicircular glass plates whose diametrical surfaces are optically worked and maintained in close mutual contact. A quadrant of one of the plates is optically worked to form a wedge having an angle of approximately 10 degrees (fig. 1). The semicircular elements are cemented with Araldite to a glass base plate having a hole of about 5 mm radius concentric with the compound disk formed by the diametrically-contacted elements. This design makes it possible to avoid spoiling the diametrical interface with surplus cement, because the cement is confined to the perimeter of the compound disk. A wedge of liquid is obtained by placing approximately 0·1 ml of the liquid under a cover-glass on the disk.

The one flat half of the glass disk will serve as the reference area and the cut edge as a sharp boundary. As the depth of the liquid is continuously increasing along the wedge, a system of evenly spaced fringes will be observed when the glass disk is viewed through the microscope in monochromatic light (fig. 2, A). The thickness of the glass disk necessitates an objective of sufficiently long working distance; the 10× objective is therefore used. The length of the fringes will be 330 μ, which is the lateral dimension of the shearing area of this objective.

A fringe will appear whenever the optical path difference between the reference area and the liquid increases by one wavelength. If the layer thick-

[Quarterly Journal of Microscopical Science, Vol. 98, part 2, pp. 151–154, June 1957.]

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ness of the liquid at the place of the first fringe is t, the next fringe will appear at the place where the layer thickness is $2t$, the next at $3t$, &c.: so the difference in layer thickness between two consecutive fringes is t. The numerical value of t depends upon the difference between the refractive index of the glass n_g, and that of the liquid n_m. The greater this difference is the smaller is the numerical value of t and a greater number of fringes per unit length is observed. When $n_g - n_m$ decreases fewer fringes will appear and

A   B
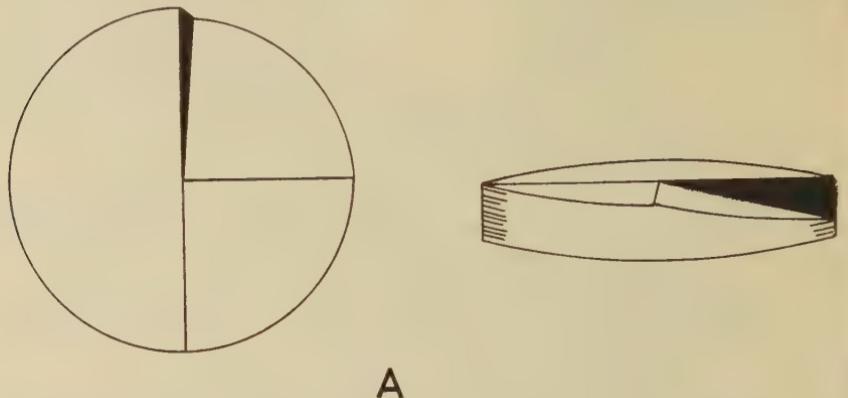
FIG. 1. Refractometer seen from above (A) and from the side (B).

when $n_g = n_m$ no fringes will be seen. If we call the distance between two consecutive fringes d and the number of fringes per unit wedge-length NF we have

$$(n_g - n_m) \simeq \frac{1}{t} \simeq \frac{1}{d} \simeq NF. \qquad (1)$$

Fig. 2, B depicts the appearance of the fringes for liquids of different refractive indices.

The measurement of the refractive index of a liquid is carried out either by measuring the distance between two consecutive fringes or, as we prefer, by counting the number of fringes per unit length, the fractional part of a fringe being measured by the use of the phase-measuring goniometer of the microscope. Fig. 3 shows the results of a series of measurements of liquids of different refractive indices. As seen from this figure—and from equation (1) —there is a linear relationship between the refractive index and the number of fringes per unit length.

As the refractive index of the glass will be known—1·5193 for this particular glass disk—and at this point the number of fringes is zero, the calibration of the refractometer is very simple. All that is needed is to determine the number of fringes per unit length for air ($n = 1·0000$) and then in a coordinate system, where the number of fringes per unit length is the ordinate and the

FIG. 2 (plate). The appearance of fringes when the refractometer is filled with castor oil (A) and with various liquids (B).

A
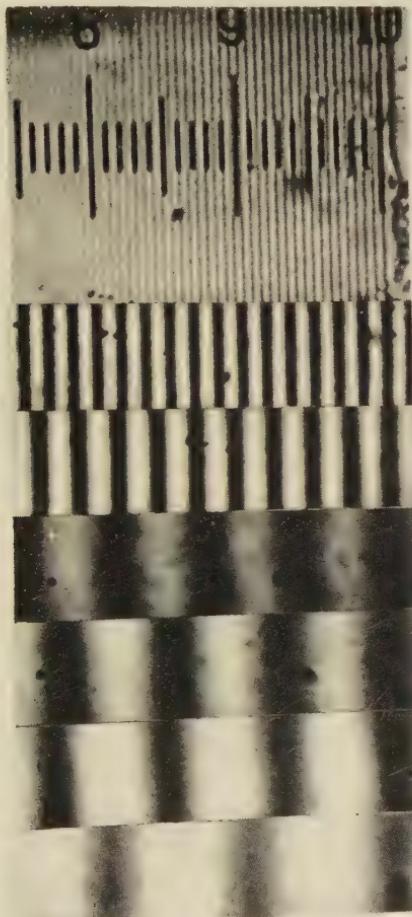
Air

Water

Paraldehyde

Glycerine

Castor oil

Liquid paraffin

Amyl pthalate

B

FIG. 2

S. IVERSEN *and* F. H. SMITH

No. of fringes
  per O·1mm
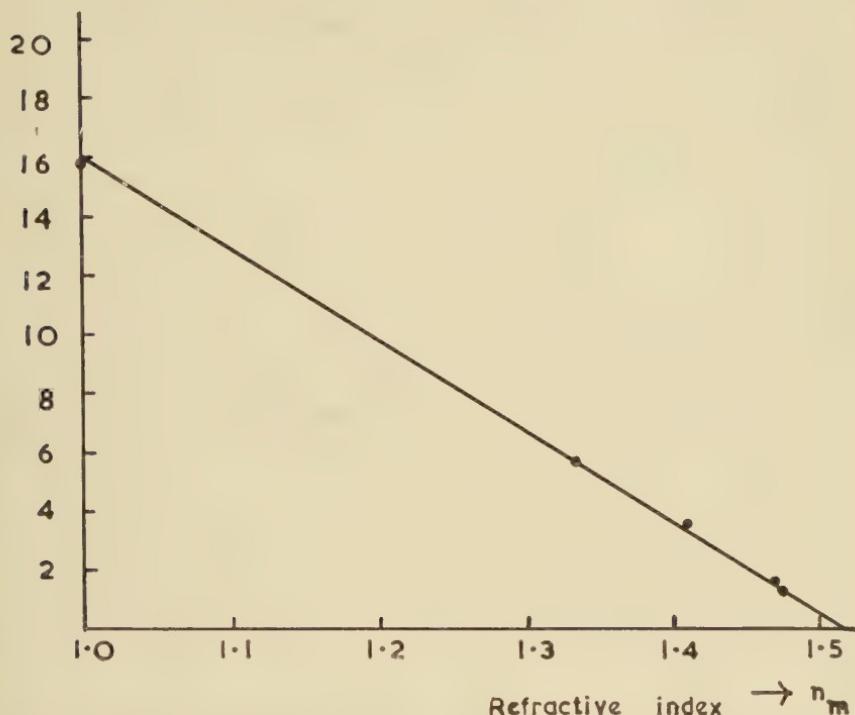
FIG. 3. Graph showing the relation between the number of fringes per unit distance and the refractive index of the medium.

...efractive index the abscissa, to connect the two points by a straight line. ...Where greater accuracy is required, arithmetical computation is preferable. ...f A is the number of fringes in air per unit length, we have

$$\frac{A}{n_g - 1 \cdot 0000} = K, \qquad (2)$$

...nd if N is the number of fringes per unit length for a liquid of unknown ...efractive index (n_m), we have

$$\frac{N}{K} = n_g - n_m, \qquad (3)$$

...rom which, as N, K, and n_g are known, n_m can be calculated.

As the true value is always unknown, the accuracy of a measurement is a ...matter of probability. As the degree of accuracy obtainable with this refracto- ...meter obviously depends upon the accuracy with which the fractional part of ...ringes can be determined, the standard deviation from a series of determina- ...ions of fractional fringes will be used as a measure for this probability.

Fig. 2, B shows that the width of the fringes increases as the difference between n_g and n_m decreases, and it might therefore be thought that the precision with which fractional fringes could be determined would depend upon the fringe width. That this is not the case is evident from results of measurements of fractional fringes in water and liquid paraffin. From 10 determinations in water the mean was found to be 0·4161 fringe with a standard deviation (s) of 0·01561 fringe and for liquid paraffin the mean was 0·1506 fringe with a standard deviation of 0·01214. As the variances of the two sets do not differ significantly ($F = 1.65$; $0.7 < P < 0.9$), it can be concluded that the fringe width has no effect upon the precision. Further five sets of 10 determinations of fractional fringes for different liquids—at room temperatures ranging from 20° C to 25° C—showed, as judged by Bartlett's test, identical variances. These were therefore combined, yielding a standard deviation of 0·01428 fringe. As the mean value of a normal distribution is being considered as the best estimate of the true value, this numerical value of the standard deviation means that in 99 out of 100 cases a single measurement will deviate 0·001 or less in refractive index from the mean, or true value. This accuracy can be improved upon by repeating the measurement. If, for example, two determinations are carried out, either by repeating the measurement on the same unit length or by measuring on two unit lengths, the deviation from the mean would be 0·0007 or less in refractive index.

The accuracy obtainable with this instrument compares favourably with the accuracy obtained by other micro-methods (Bauer, 1949; Jelley, 1949) where the accuracy varies between 0·005 and 0·0005 in refractive index.

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A Note on the Opacity to X-Rays of Tissues fixed with Mercuric Chloride

By G. E. H. FOXON

(*Department of Biology, Guy's Hospital Medical School, London, S.E. 1*)

With one plate (fig. 1)

SUMMARY

The radiography of 'soft' tissues normally requires special X-ray apparatus which generates soft X-rays. Fixation with mercuric chloride, either alone or in mixture, has been found to render such 'soft' tissues opaque to those harder X-rays generated by normal clinical X-ray apparatus. Radiographs made by the use of such standard apparatus may be useful in several fields of biological research such as anatomical investigation and illustration, or in the study of the penetration and behaviour of the fixatives themselves.

IN work previously recorded (Foxon and Rowson, 1956) the fate of the radio-opaque medium, thorotrast, injected into the dorsal lymph space of the frog was followed. The methods used sometimes necessitated the removal, from a freshly killed frog, of the alimentary canal together with the liver and spleen which were then radiographed separately from the rest of the body; afterwards the alimentary canal and associated glands were fixed and sectioned by the usual methods, and the appearances seen in the radiograph were interpreted in the light of evidence provided by a study of the sections. On one occasion after an alimentary canal had been placed overnight in Zenker-formaldehyde solution it was decided that it should be radiographed again. Whereas usually only those parts of the preparation containing thorotrast had shown in the radiograph, on this occasion it was found that the whole alimentary canal had given X-ray shadows of remarkable clarity, the folds of the mucous membrane in the intestine being especially conspicuous. Such a radiograph is shown in fig. 1, A.

It was realized that this curious result had been obtained because mercuric salts had been absorbed by the tissue of the alimentary canal, and this was confirmed by attempting to radiograph specimens fixed in a non-mercuric fixative such as Bouin's fluid, which gave no result, and also by using mercuric chloride both with and without acetic acid. These two fixatives gave similar but not superior radiographs to Zenker-formaldehyde.

This property of fixatives containing mercury does not appear to have been recorded before. It would seem to be of importance from two points of view. First, because the affinity between the mercury and different tissues seems to be unequal, it is possible to produce an effect akin to 'differential staining'; and, second, because it would be possible to use this method to investigate the

penetration or, alternatively, the removal from tissue of mercury-containing fixatives.

The 'differential staining' effect just referred to is well shown in a radiograph of a chick-embryo (fig. 1, B), in which it is very remarkable how the lining of certain of the blood-vessels of the area vasculosa have cast very marked shadows. In Platyhelminthes the alimentary canal seems to show well and sometimes the reproductive organs (fig. 1, C–E). Such radiographs as these may be useful as methods of anatomical investigation and illustration.

Radiographs of 'soft' tissues have been found useful by many workers, but, as is well known (see, for example, Barclay, 1951), only very 'soft' X-rays (i.e. those generated by X-ray tubes using a comparatively low voltage such as 10 or 12 kV) are of use. As these soft X-rays are harmful to human skin and also lack power of penetration, they are purposely not generated in X-ray apparatus designed for medical work, but it is usually such apparatus that is available to biologists for research purposes. With these X-ray sets it is unusual for a tension of less than 30 kV to be available and the rays produced are much too hard for soft-tissue work. However, the method described here overcomes this difficulty by making the soft tissues much more radio-opaque, and thus kilo-voltages normal in diagnostic X-ray apparatus can be used. Whether the information conveyed by such radiographs is of value can only be judged in particular instances, but, if it is desired to compare the branching of the alimentary canal in a large number of liver-flukes or planarians, radiographs can be made of several on one plate or film only 24 hours after fixation. By the usual methods, the specimens would have to be carefully stained and mounted and then photographed separately; and to build up a collection of a large number of photographs would take an appreciable time.

The technique now used is as follows: fixation in Zenker-formaldehyde is carried out in the normal way for 24 hours; if not required for immediate use, the material can be stored in a solution of mercuric chloride 5 g, potassium

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Fig. 1 (plate). Positive enlargements of radiographs showing:

A, portion of intestine of frog. (Fixed Zenker-formaldehyde. Ilford Process plate. 200 mA. 30 kV 2·25 sec. Focus/film distance 50 cm. Developed I.D. 2 diluted 1 to 2 of water, 6 min at 70° F.)

B, blastoderm with embryo chick. (Fixed Zenker-formaldehyde. Ortholine Cut-Film. 100 mA. 30 kV 5·25 sec. F/f distance 50 cm. Developed as A.)

C, a planarian. (Anaesthetized in chloretone. Fixed Zenker-formaldehyde. Ortholine Cut-Film. Exposure and development as B.)

D, a sheep liver-fluke. (Fixed Zenker-formaldehyde in November 1955; radiographed 21.ii.56. Ilford Process plate. 100 mA. 30 kV 4·5 sec. F/f distance 50 cm. Developed as A.)

E, two proglottids of a human tapeworm. (Fixed in formalin, subsequently soaked in Zenker-formaldehyde for 5 days. Ortholine Cut-Film. 100 mA. 30 kV 6·5 sec. F/f distance 50 cm. Developed as A.)

F, the anterior part of an earthworm in side view. (Worm killed in alcohol, then fixed in Zenker-formaldehyde. Ilford Process plate. 100 mA. 40 kV 3·5 sec. F/f distance 50 cm. Developed as A.)

G, a dissection of the anterior segments of an earthworm, dorsal view. (Worm killed in alcohol, then dissected and then soaked in Zenker-formaldehyde for 24 hours. Ilford Process plate. 100 mA. 35 kV 4·5 sec. F/f distance 50 cm. Developed as A.)
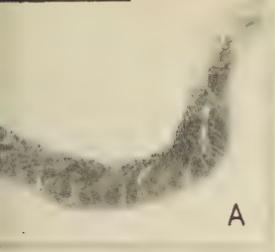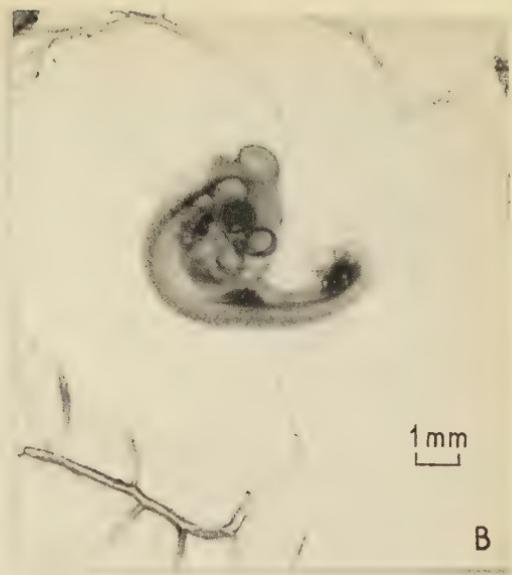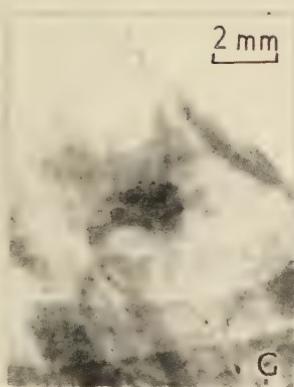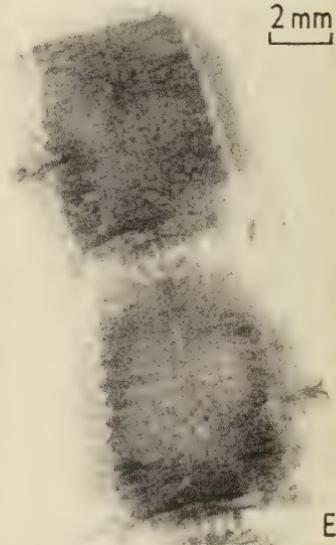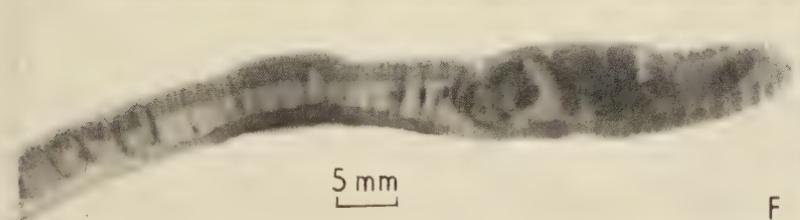
Fig. 1

G. E. H. FOXON

dichromate 2·5 g, sodium sulphate 1 g, distilled water 100 ml. Successful radiographs of small objects, such as chick-embryos, have been made of material stored in this way for up to three weeks; after this time such specimens become so brittle that manipulation of them becomes impossible. There is also a marked tendency for the radiographs to become spotted, as if the mercury were accumulating in small granular masses. With larger specimens more prolonged storage is possible, and the liver-fluke shown in fig. 1, D had been stored for three months before it was radiographed. Radiographs are usually made, therefore, as soon after 24 hours' fixation as possible.

When a radiograph is to be made, the specimen is washed in tap-water. In most instances this wash has been rapid with the intention of removing excess fixative from the surface of the specimen. A preliminary experiment has indicated that more prolonged washing gives greater differentiation between various tissues, but further experiments would be needed to demonstrate how useful this process might be.

As sensitive materials, Ilford Process Plates and Kodak Maximum Resolution Plates have been used, and also Ilford Ortholine cut-film. The plate or film is enclosed in a light-tight cardboard box, a sheet of lead to prevent back scatter of radiation being placed under the plate. The plate is laid emulsion-side upwards. As recommended by Barclay (1951) for micro-arteriography, a sheet of Styrafoil 'S' 1/1000 in. thick is laid on the surface of the plate and the specimens to be radiographed laid on the Styrafoil by means of a camel-hair brush, care being taken to remove as much of the water from the surface of the specimen as possible. As also recommended by Barclay, if the exposure is to be long (as with Maximum Resolution Plates, where it may have to be built up over half an hour or more, the tube being rested between exposures), a second layer of Styrafoil is laid over the specimens to prevent drying, but this increases the exposure time. Up to this point the procedure has been carried out in the dark room, with the appropriate safe-light. The lid of the box is now placed in position and the whole preparation transferred to the X-ray set with as little disturbance as possible and the exposure made. The radiographic factors used for the illustrations of this paper are given in the legend to the figure.

One modification of this technique which has been tried may be useful. Proglottids from a human tapeworm, which had been fixed for 48 hours in ordinary 5% formalin, were washed in water and then soaked in Zenker-formaldehyde for 5 days, after which radiographs were made. The results are shown in fig. 1, E.

Fig. 1, F, G shows how the anatomy of a larger animal, an earthworm, may be illustrated by this method.

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Internal Structure of Avian Melanin Granules: An Electron Microscope Study

By J. G. CARR

(From the British Empire Cancer Campaign Unit at the Agricultural Research Council Poultry Research Centre, Edinburgh, 9)

With one plate (fig. 1)

SUMMARY

The black melanin granule of the Brown Leghorn male is a separate particle of complex structure, differing from the brown granules of the female, chick down, and Rhode Island Red.

It is feasible to study the granules in the electron microscope directly in the intact feather.

MELANIN granules represent an interesting type of separate and character-istic intracellular particle formed by specific cells. While much attention has been devoted to the chemistry of the formation of the pigment, rela-tively little has been directed to the particles themselves, probably because they lie on the border of resolution of the optical microscope. Capacity to secrete these granules may sometimes be lost, but is regained by a mechanism which has some analogies to an infective process. This has been described for grafts of mammalian skin by Billingham and Medawar (1948), and a somewhat similar process may have occurred in the case of human melanoma described by Botha and Lennox (1954). In their case the melanoma metastases, as often happens, had lost the ability to form pigment, but this had been regained when they came into contact with the skin. Unlike the plant enzyme, mammalian tyrosinase is associated with large ultramicroscopic particles, which may even be the actual melanin granules themselves.

It is known that melanin particles may differ in shape in various species, but their exact differentiation from adventitious dirt is not easy. This is shown, for example, in the account of the granules in various breeds of fowl by Boh-ren, Conrad, and Warren (1943). Mason and his colleagues studied mamma-lian granules (from cattle, mice, and man) by the electron microscope, and concluded that they were definite 'formed elements' but that they had no internal structure; no great differences in size or shape were found among the various granules.

An investigation of the melanin granules of the fowl by the electron micro-scope showed that, unlike the mammalian particles, some of them had a com-plex internal structure, and the various types differed very much in both form and size. Furthermore, it has been found possible to examine the particles directly in the feather by the electron microscope. This opens the way to a study of the genetic and racial variations, as well as the endocrine-induced changes, by morphological means. As it is not intended to pursue this type of study, a description of the methods and results so far obtained may be of value to other workers.

<center>RESULTS</center>

Examination of free particles

Either skin with growing feathers, clipped free of fully formed feather, or the roots of growing feathers plucked from the skin were used. The material was macerated with saline and the particles concentrated and washed by centrifuging; the coloured layer of granules was easily followed through the processing. After washing and purifying by a few cycles of centrifuging, a suitable dilution was mounted on electron microscope grids covered with collodion in the usual way. Examination was by a Philips 25A electron microscope; any shadowing was done with gold-palladium alloy.

The particles from the black breast feathers of the male Brown Leghorn were found to consist of dense rounded rectangles or lenticules about 1 μ long, with a smooth and sharply defined edge and surface. They have a well-defined exterior wall, about one-quarter of the breadth of an average granule; this is quite structureless. The central space may contain, rarely, a few 'vacuoles', but is more usually filled with a single row of reticulated material (fig. 1 A), or sometimes two or three rows (B). This latter condition is usually seen in the more lenticular granules; it gives a central, or more infrequently subterminal, bulge (C). Occasionally the central space seems almost empty, containing only a few thin transverse strands. Shadowed preparations indicate that the surface is smooth, and that the granules are also lenticular in cross-section, with the greatest thickness about one-half of the breadth. Sometimes abnormally long forms are found. These appear to be almost double granules, arranged end to end (D), perhaps with only a small portion of the structureless wall in common. These forms suggest either multiplication by self-reduplication or in sequence from a generation point. The extreme diversity of forms was quite striking.

That this internal structure is not an artifact of preparation is indicated by the fact that exactly similar pictures were obtained when the extracted material was immediately fixed by buffered formalin or osmium tetroxide before separation, and also by the results of direct examination of the feathers, as described below.

Examination of formed feathers

It was found, rather surprisingly, that the particles could easily be examined directly by the electron microscope in fully formed feathers. A single barb or barbule is washed in acetone, dried, and mounted across a 100-mesh copper

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FIG. 1 (plate). A and B, black melanin from Brown Leghorn male. Shadowed; gold-palladium.

    C, the same, but unshadowed.

    D, joined granules, shadowed.

    E, feather containing black melanin granules.

    F, Rhode Island Red granules, unshadowed.

    G, shadowed; gold-palladium.
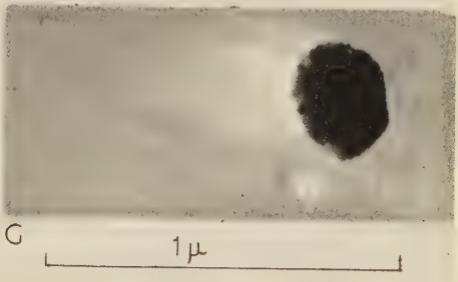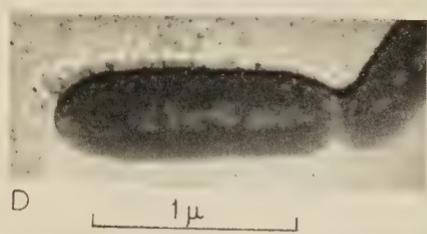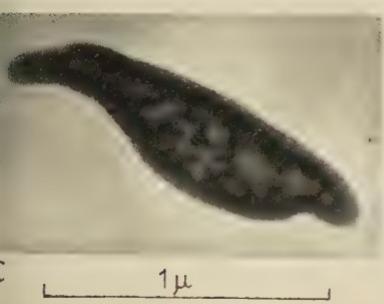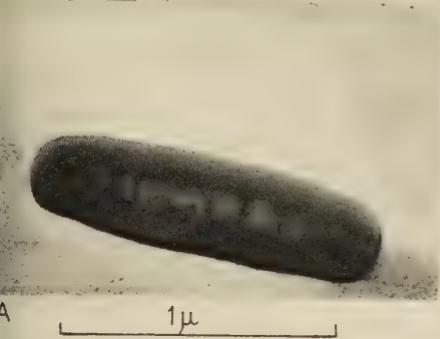
FIG. 1

J. G. CARR

grid. When inserted into the microscope vacuum, the feather 'explodes' and shears along the keratin layers. Further shearing occurs in the heat of the electron beam, leaving the granules mounted for examination on a very thin layer of keratin that is relatively transparent to the electron beam and almost structureless, at the magnifications used. Naturally, under these conditions the resolution is not very good, as the static charges on the feather cause some distortion of the electron beam, and also the preparation is not usually perfectly stationary. Details of the interior of the black granules can still be made out, however, and the actual preparations, examined with continual adjustments of focusing and beam-centring, give a much better idea of the structure than the photograph (fig. 1, E) indicates, since the exposure needed for photography is too long for the unstable conditions of the preparation.

Observations by this method of the black male feathers confirmed that the smooth surface, internal structure, shape, and thickness were as deduced from the shadowed preparations of free particles. An impression was gained that the particles, arranged in lengthwise rows, contributed quite notably to the bulk and strength of the feathers.

White feathers, or patches of white found in feathers, often occurring in fowls, were found to be quite devoid of granules. Occasional inclusions of dirty material were quite easily distinguished from granules.

Rhode Island Red feathers examined by the same direct method contained granules which were obviously quite different in structure. These were discoidal, and with no internal structure, very uniform in size, and closely resembled the mammalian granules described by Mason and his colleagues (1947). Not one looking like the black male Leghorn type was seen. The down and female breast feathers of Brown Leghorns contained both types of granules, but the various areas examined were usually clearly of one type or the other, and not mixed.

When examined as a preparation of free particles, Rhode Island Red granules were seen to be roughly rounded or comma-shaped particles, about one-third of the length of the black Brown Leghorn ones, having a rather rough crenated wall and only a hint of an internal structure (fig. 1, F, G). Compared with the former they strongly suggested a badly made and imperfect product, which is not surprising as they are the end-result of a mutation from the normal. As in the case of the black granules, they were arranged in the feather in long strands, with the edges almost touching.

Needless to say, this method of 'exploding' the feathers in the microscope resulted in a certain amount of debris being left in the instrument. On cleaning, numerous fragments of feather were found in the object cavity. Fortunately, the Philips microscope is very tolerant of dirt in this position, though its performance was doubtless somewhat impaired by the presence of this material. No feather particles were found elsewhere. However, as the objective of the Philips microscope can only be cleaned after a complete dismantling of the whole microscope column, it seems preferable that another type of instrument should be used for such work.

Discussion

The secretion of melanin pigments in the fowl in the form of granules is clearly an elaborate process terminating in the formation of a particle of complicated structure, and not merely the production of an insoluble lump of protein-melanin complex. The change from black to red—which is under hormone control—is not merely one of chemical structure, but also of the gross structure of the granule itself. It would therefore appear that the avian material offers especially favourable material for such studies; for the basic type of granule is a well-defined and easily recognizable structure, differing from any other reported type of cell organelle. The problem of the extra-epidermal origin and the development of granule-secreting properties in the cells of the neural crest of the embryo (Dorris, 1945; Rawles, 1945) can thus be favourably studied in well-known and easily available material. The fact that avian granules have a recognizable and characteristic structure suggests that further comparative studies should be interesting. Melaninization is found in most of the Metazoa, and a study of this by micro-morphological methods may be worth while. Methods analogous to those described for the use of very small pieces of whole feather could doubtless be used on museum specimens without causing any particular damage.

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Mauthner Neurones in Young Larval Lampreys (*Lampetra* spp.)

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With two plates (figs. 6 and 7)

SUMMARY

'Mauthner's cells' are a pair of very large neurones found in the hind-brain in all main groups of fishes and amphibia, except in sharks and rays. Mauthner cells always have some dendrites in synaptic relation with incoming VIIIth-nerve fibres and have a large axon which crosses the brain and descends the opposite side of the spinal cord, co-ordinating somatic motor activity. Papers discussing the presence of these cells in the lampreys are reviewed: two different pairs in the lamprey of 'Müller's cells' (giant co-ordinating cells with homolateral axons) have been considered homologous with Mauthner cells, but recent textbooks accept neither homology.

A pair of very large neurones, having the Mauthnerian characteristics, is described in the embryonic and early larval stages of *Lampetra planeri* and *L. fluviatilis*. The neurones are illustrated by figures in different planes. They are not one of the pairs of Müller neurones described by previous workers.

From this and similar evidence it is suggested that in all non-amniote vertebrates the earliest swimming movements under the control of the brain are effected through variations from one homologous co-ordinating system, of which the Mauthner cells form part.

CONTENTS

INTRODUCTION

AN earlier generation of zoologists sometimes grouped together the more primitive, aquatic vertebrates as the 'ichthyopsids'. The term covers lampreys, cartilaginous and bony fishes, the tadpole stage of amphibians, and adults of aquatic tailed amphibians. These animals have certain significant characters in common; in particular, they possess a lateral-line sensory system and their locomotion is achieved typically by serial contractions of trunk and tail myotomes.

The organization of neurones and of somatic muscle which brings about the swimming movements of ichthyopsid animals may therefore be homologous in them all. If this is so, a close comparison between different members of the

group is justifiable, and should give valuable information on several aspects of the working of the nervous system.

On the other hand, it may be that the developmental sequence by which the swimming mechanism is achieved has followed different paths. Even within an homologous morphological system of myotomes, peripheral nerves, and central tracts, the neurones forming the units of this system may have combined into functional patterns which, although they all provide for swimming movements as part of the behaviour pattern, have evolved independently in different groups, e.g. in the different vertebrate classes.

It is therefore necessary to see whether there exists a sound basis for detailed comparison of neurones and sensori-motor arcs among the ichthyopsids.

The simplest living example of the ichthyopsids is the newly hatched or 'pro-ammocoete' larva of the lamprey. The lampreys, although of cephalaspid rather than pteraspid affinities, have probably diverged little from the structure of the agnathan ancestor of the jawed vertebrates. The organization of the cranial and spinal nerves and of the gross morphology and tract-systems of the lamprey brain appears to be primitive among vertebrates, with few specializations: this has been shown especially by the work of Johnston (1902, 1905, 1912). The pro-ammocoete is without some specialized features of the ammocoete and adult stages: its brain is not yet compressed from in front by the backward growth of the naso-hypophysial tube, the eyes and eye-muscles are not degenerate, and the spinal cord is cylindrical, and not ribbon-shaped as in the ammocoete or adult.

The neuromuscular organization of the trunk of the pro-ammocoete is comparable with that of higher ichthyopsids such as the larvae of urodele amphibians on which Coghill made his now classical researches on the relation of behaviour with neuromuscular anatomy. The neurones which could effect a contralateral response develop very early, as they do in urodeles. These neurones are (a) *Rohon-Beard somatic-sensory* cells (these resemble dorsal-ganglion cells but the cell-body lies within the cord); (b) *'primary' somatic-motor* neurones; (c) *large internuncial* neurones providing a contralateral linking of the sensori-motor pattern. Types (a) and (b) are strikingly similar to the corresponding types described by Coghill. I have described this organization in the pro-ammocoete previously (Whiting, 1948). The physiology of the neuromuscular system also seems to be similar to that of higher ichthyopsids (compare Harris, 1955).

It seems probable that a contralateral sensori-motor arc, developing first at a post-otic level and then rapidly at successive cephalo-caudal levels, is the first functional system of neurones in all ichthyopsids. The facts, on which this view is based, are given elsewhere (Whiting, 1955). Now, in the lamprey embryo, as cells of the types (a), (b), and (c) develop at more caudal levels of the cord, so there descend to these levels the growth-cones of axons from the first co-ordinating cranial neurones (Harris and Whiting, unpublished). These neurones, when functional, will bring the movements of the somatic muscle under the control of the brain; they are known as the giant *Müller neurones*; the cell-bodies, of which there are several pairs, lie in the mid- and hind-brain

loor and none of the axons cross the mid-line as they descend the spinal cord.
n higher ichthyopsids, on the other hand, growth down the cord of axons of
o-ordinating neurones occurs relatively later, and one pair of axons is parti-
ularly prominent. These arise from a pair of giant *Mauthner* neurones.

After comparison of the earliest sensori-motor arcs ((*a*), (*b*), (*c*)) of the trunk
f the lamprey with those of higher vertebrates, it is important to establish
vhether the brain of the lamprey then co-ordinates spinal cord activity in the
ame way as in higher animals. The Mauthner neurones provide an important
lement in this second step.

The Mauthner pair has many distinguishing features: the cell-body lies
t the level of the otic-capsule, certain constant relations of a few prominent
dendrites include a direct synaptic connexion with incoming fibres of the
VIIIth nerve, the axon, of outstanding diameter, *crosses* the floor of the hind-
brain to descend the length of the cord in synaptic relation with the *contra-
lateral* primary somatic-motor neurones. Intensive study, by Beccari, Bartel-
mez and Hoerr, Detwiler, Stefanelli, Bodian, and Leghissa among many
others, has revealed important structural and developmental characters of the
Mauthner neurones. This information has helped our understanding of ner-
vous function, and has shown, I think, that the Mauthner apparatus is a part
of a specific co-ordinating mechanism, much as a well-defined pattern of key
indicates the existence of a specific lock. This specificity is indicated also by
the existence of many other pairs of co-ordinating neurones which, while less
striking, are equally constant in position (compare Bartelmez, 1915, on
Amiurus, and Stefanelli and Camposano, 1946, on *Anguilla*).

Functionally, these cells have been shown to play a part in the development
of effective swimming movements in the larval stages (Detwiler, 1933). The
Mauthner cell is important in the movements of the tail by which the animal
maintains equilibrium: the connexions of one of its dendrites with the vesti-
bular division of the VIIIth nerve suggests this, as does the absence of the
cell in those fish in which the tail is lost (*Mola*) or does not assist in equili-
brium (some bottom-living fish). Control of the tail cannot be its only func-
tion, since it is in synaptic relation with motor neurones at all levels of the
cord. More detailed information on the functions and connexions of the
Mauthner cell is given by Kappers and others (1936), Beccari (1943), Piatt
(1948), Leghissa (1946, 1947), Stefanelli (1951), Bodian (1952), and Cordier
(1954). Cordier's figures (pp. 250 and 327) give the general relations of the
cell to the rest of the brain in an adult fish or amphibian.

Mauthner cells have been described in the holocephalan *Chimaera*, in the
lungfish *Neoceratodus*, in the sturgeons *Acipenser* and *Polyodon* (Johnston,
1901, Hoogenboom, 1929), in the great majority of the teleost fishes which have
been examined, in the larval stages of urodele, anuran, and apodan Amphibia,
and in adult aquatic urodeles. They are found in a reduced form in some adult
frogs. They have not been found in elasmobranchs, whether sharks or rays.
The most complete survey of their distribution is given by Beccari (1943),
who, however, omits the sturgeons from his list.

It is therefore important to know whether Mauthner neurones, in additic
to Müller cells, exist in lampreys. For the presence here of Mauthner cel
would then suggest that the whole mechanism by which the brain co-ordinat
the activity of the spinal cord is homologous in all ichthyopsids: their absen
from elasmobranchs would be secondary. It would become justifiable to a
tempt a detailed comparison not only of the behaviour but of the senson
motor arcs and the cell-types found in the development of all ichthyopsids u
to the relatively late stage at which swimming locomotion under the contr
of the brain is achieved.
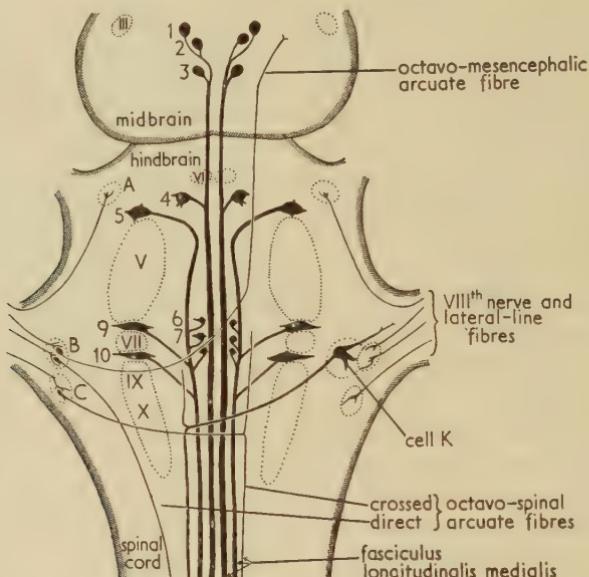
FIG. 1. Diagram of part of brain of *Petromyzon marinus*, showing the relation-
ships of the large co-ordinating neurones. After Stefanelli (1934, fig. 6), with
some detail omitted and a neurone *K* added. A, B, C, *anterior, ventral, posterior*
acoustico-lateral *nuclei* (*medial* and *dorsal* centres, at level of *ventral* nucleus, not
shown). Roman numerals: motor centres of cranial nerves. Gothic numerals:
Müller cells (Stefanelli's numbering). *K*, hypothetical giant cell in ventral
nucleus (expanded).

The studies of neurones of the central nervous system of the lamprey
which have been made over the past 80 years, might be expected to hav
settled this question a long time ago, but in fact the results have been ver
conflicting. The present account provides evidence that the pro-ammocoet
stage of the lamprey does possess true Mauthner neurones.

Previous Work

Ahlborn (1883), in surprisingly detailed work for the period, found sever
pairs of Müller neurones and one pair of Mauthner neurones in young ammo

oetes of 15–20 mm length. His plan-view diagram shows the Mauthner
ell-bodies at the level of the VIIIth nerve and the Mauthner axons forming
chiasma and then descending the spinal cord on the side opposite to the cell
f origin.

Since then there have been many accounts of the co-ordinating apparatus
n the brain of the lampreys; fortunately, descriptions of the Müller cell part
f it agree upon the facts. In older ammocoetes and in adults, the organiza-
on of the Müller cells has been found to have a well-defined pattern of the
orm shown in plan view in fig. 1, which follows Stefanelli (1934). The pairs
f Müller cells *9* and *10* in this figure are the important ones for the present
roblem. The neurone *K* is not part of the known co-ordinating system but is
 hypothetical cell which will be discussed later. The Müller cells are shown
n lateral view in the book by Kappers and his colleagues (1936, fig. 316),
here cells *9* and *10* are the upper two at position 'B'.

The shape and position of each Müller cell-body is constant within a species,
nd readily comparable between species; the position is either along the
iscero-motor column, or, more medially, on the somatic-motor column.
hese results have been established by the work of Johnston (1902), Tretjakoff
1909), Saito (1928), Stefanelli (1934), Pearson (1936), Woodburne (1936), and
arnard (1936). It is agreed that each Müller axon runs along the spinal cord
n the same side as the cell-body from which it comes. Tretjakoff has men-
oned that some neurones of the medial group have processes, apparently
xon-collaterals, which cross the hind-brain floor to form a commissure, the
hiasma fibrarum Müllerianum; but Johnston (1910) reaffirmed that no Müller
xons decussate in the hind-brain.

A typical Mauthner cell with a contralateral axon, as described by Ahlborn,
as not found again until Whiting (1955) briefly stated that a homologue of
ie Mauthner neurone exists in the lamprey embryo and showed, in a diagram,
ie relative positions in the spinal cord of the Mauthner and Müller fibres.

Other recent work on the nervous system of the head of the lamprey
eghissa, 1942; Larsell, 1947; Heier, 1948; Lindström, 1949) does not give
ny new information about the Mauthner or Müller cells.

MATERIAL AND METHODS

Early motile stages of *Lampetra planeri* and *L. fluviatilis* were used. No
ifferences were found between the two species. Specimens were taken from
ggs which had been fertilized on the nest or in the laboratory. Serial sections
f many embryos and larvae were prepared; over 20 specimens were carefully
udied in working out the connexions described below.

The stages of development were determined by the criteria given by Damas
1944). Observations were made on animals of 3·5 to 8·0 mm in length, i.e.
otile stages of the embryo, stages 7 to 11 of Damas; newly hatched larvae,
ages 12 to 14; and an immediately following stage, larvae of 6·5 to 8·0 mm
 length, which may be termed stage 14*a* since it is much younger than

Damas's stage 15. The 8-mm larvae still have well-developed eyes, a cylindrical spinal cord, and a naso-hypophysis opening at the front of the head (not dorsally); in the free state they would not yet have burrowed into the bed of the stream: they have not changed from 'pro-ammocoetes' into prides ('ammocoete' larvae).

Impregnation of nervous structure was effected by Bodian's protargol or Holmes's silver nitrate techniques used after Nonidez's fixative and Lang's method for dehydration (Whiting, 1948; Harris and Whiting, 1954). It was difficult to get good impregnation of thin sections but these were easier to study; the most useful were 8 μ in thickness. Non-nervous detail is shown well in embryos fixed in Susa, dehydrated by Lang's method and stained with Mallory's phosphotungstic acid haematoxylin, but the histology of muscle- and nerve-cells is shown in good protargol preparations.

Preparations of the brain of adult *Lampetra* were compared with the account by Johnston (1902). His 19 figures of transverse sections of Golgi preparations were photographed and set up as a three-dimensional model, with the functional components traced out in threads of different colour.

Final stages of the work were done with camera lucida drawings made with a Zeiss-Winkel drawing apparatus, which is accurate over its whole field: in this apparatus the relative brightness of the drawing surface is varied by means of two rotatable polarization filters. Nerve-processes can be traced from the lowest optical plane of one section to the highest plane of the succeeding section by this means, if the points of reference for the figure are well chosen. This procedure can be repeated through many sections. Drawings of important series of sections were made on sheets of Kodatrace, and fibres were followed on these.

PERSONAL OBSERVATIONS

The organization is best seen at about stage 14. Where convenient, earlier stages are referred to, as well as the time at which cells, once identified, can be first found.

In transverse sections of stages 14 and 14a, a pair of very large fibres was observed to cross the floor of the brain at a level slightly posterior to the otic capsules, in a marked and symmetrical chiasma. The fibres were difficult to follow forward, in sections cut in the usual planes, because they turned obliquely upwards and outwards. A series of sections cut parallel to the long axis of the animal, but at about 35° to its vertical axis, proved to be in the plane of orientation of the cell-body and of the anterior part of the axon, and is illustrated in figs. 2 and 3.

Fig. 2 shows the organization of the head at this time; the dimensions have been confirmed by comparison with larvae of the same age, cut sagittally. The arrangement of nerves and visceral arches illustrates a primitive vertebrate condition in very beautiful fashion. The profundus ganglion and nerve run anteriorly forward, dorsal to the optic nerve and eye, while the maxillomandibular nerve runs ventral to those structures. The facial nerve runs downward

ong the hyoid arch, entirely behind the first gill-pouch, which corresponds
 the spiracular pouch of fishes. The first pair of gill-pouches contains the
ir of muscular *velar folds* which can be clearly seen in rhythmical pumping
tion in the living pro-ammocoete; these gill-pouches do not form definitive
ll-openings, as do the succeeding seven pairs. Further details of the general
orphology will be found in the figure of Damas (1944, plate III, *14*).
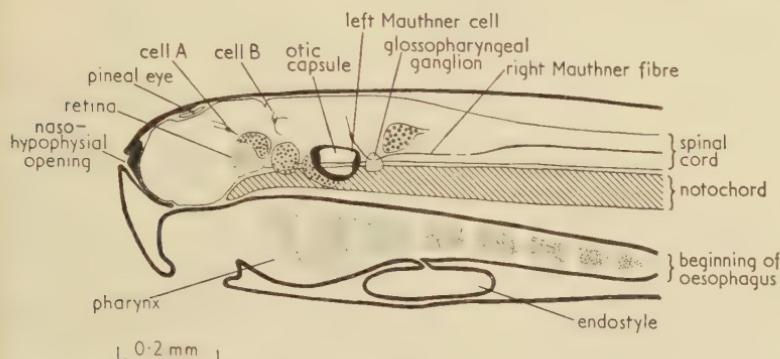
Fig. 2. Medial section, head of *Lampetra* larva (stage 14), with some lateral structures projected on to it. The brain shown in outline. Stipple: cranial ganglia (v^1, v^{2-3}, VII, and X) and gill-pouches.

 The main result may be seen from fig. 2. The cells of origin of the two fibres
 above the posterior third of the otic capsule. The axons run caudally and
edially, crossing the midline at the level of the glossopharyngeal ganglia.
ter the chiasma so formed, each of the two, now contralateral, fibres runs
nong and parallel with the ipsilateral Müller fibres (not shown) in the medial
ngitudinal bundle of that side, as far as the level of the fourth gill-pouch.
 this point two fibres, apparently the same pair of contralateral ones, swing
t of the closely packed bundle into a more dorsal and lateral position in the
inal cord and then continue caudally again, parallel with, but now distinct
om, the Müller fibres. Other series of sections confirm that it is the contra-
teral pair of fibres which swing away from the Müller fibre bundle in this
shion. The pair of cells which have this relationship are considered to be the
omologues of Mauthner cells, and will be given that name for convenience
 further reference.
 Further detail of the Mauthner cell is shown in fig. 3. Two large cells with
aracteristic dendrites lie farther forward in the brain (cell A in the mid-
ain and cell B at the front of the hind-brain): these cells and the Mauthner
ll are seen in both figures and show the relation between them. The outlines
 the brain and notochord, and the background shown within them, were
awn from a single optical level of one section of the series used in fig. 2: the
urones shown in detail, including the left *A*, *B*, and Mauthner cells, and the
ght Mauthner axon, were drawn from the whole thickness of the section.
rther detail of the most ventral part of the brain was added from the adja-

cent section on the medial side. The outlines of brain and notochord were ve[r]
similar in the two sections, except at the tip of the notochord, so that alignme[nt]
was not difficult. Cell-walls of part of the notochord were drawn from t[he]
second section *in stipple*: the stippled lines coincide, as they should if the se[c]
tions are correctly aligned, with continuous lines drawn from the same ce[ll]
walls seen in the first, more lateral section.
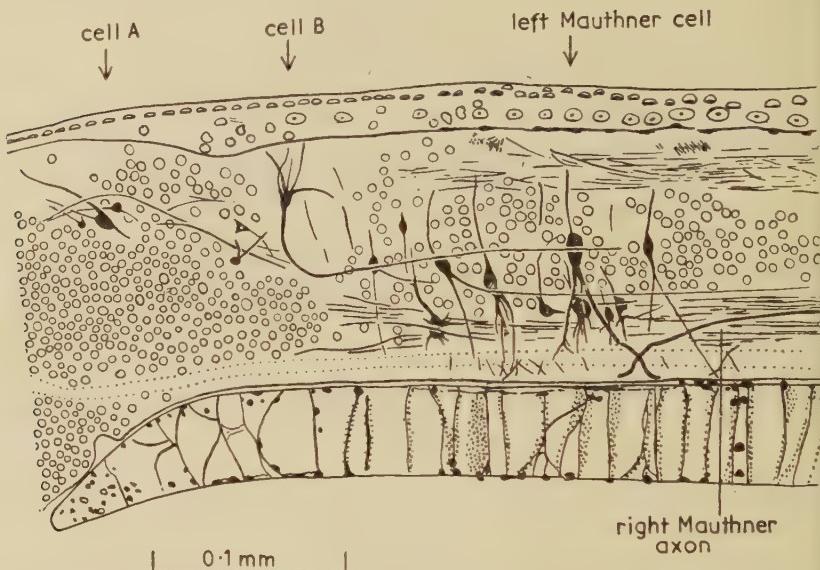
FIG. 3. Course of Mauthner axons, as they cross the mid-line; from two sections of the ser[ies]
used in fig. 2. Camera lucida drawing.

The left Mauthner axon runs caudally and ventro-medially from its ce[ll]
body, in the first section; in the next medial one, it crosses the right Mauthn[er]
fibre in the prominent chiasma seen in the figure; continuing caudally an[d]
to the right of the mid-line, it enters a third section (not drawn). From t[he]
chiasma in the second section, the right Mauthner fibre enters the first secti[on]
and joins the bundle of left-side Müller fibres, as shown.

The Mauthner cell-body lies dorsal to the nearer Müller cells, one of whi[ch]
it straddles. From the cell-body, two axon-collaterals run ventro-medial[ly]
becoming very slender as they approach the mid-line; they pass very close [to]
the outer side of this Müller cell. There are few dendrites on the Mauthn[er]
neurone, compared to the prominent array running into the white matter fr[om]
a Müller neurone: one main dendrite runs dorsally, as shown, to the point [of]
entry of the VIIIth nerve.

The figure also shows that the Mauthner neurone is, at this period, of ab[out]
the same size as the Müller neurones, but the oblique angle of its cell-bo[dy]
makes it inconspicuous in sections cut in the usual planes. This can also [be]
seen from fig. 6, A, which is of a different larva, and at a higher magnificati[on]

Fig. 4 shows the Mauthner cells of a stage 11 embryo projected, from five horizontal sections of 16 μ, on to the lowest: this passes through the middle of the otic capsules, the most anterior trunk-myotomes, and all but the tip of the notochord. The facial ganglion is still small and is ventral to the level of the section. The fourth (first post-otic) myotome is divided into a part medial
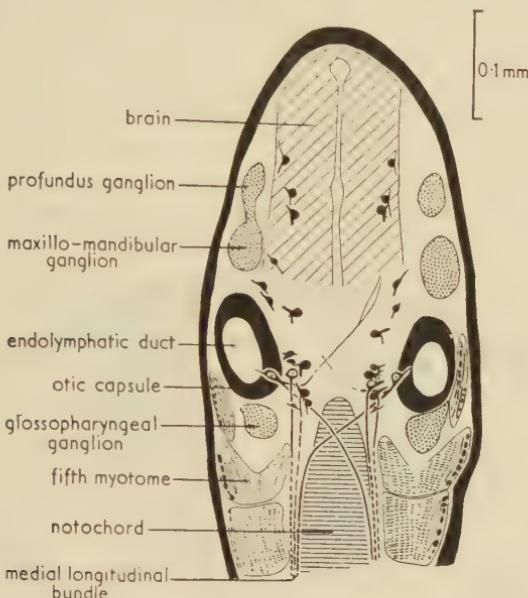
FIG. 4. From horizontal sections of a *Lampetra* embryo (stage 11); the Mauthner neurones seen from above. Camera lucida drawing.

and a part lateral to the otic capsule: the lateral part is divided into an upper (shown) and a lower half: divisions no doubt due to the relative expansion of the otic capsule. The contracting units are horizontal muscle-plates containing myofibrils; separate muscle-fibres cannot be seen (compare Brachet, 1935).

The figure shows both Mauthner cells lying obliquely, lateral to the nearest Müller cells; the Mauthner axons crossing the mid-line at the level of the glossopharyngeal ganglion, and then running caudally among ipsilateral Müller fibres; and the position of the larger Müller cells in this stage of embryo (projected from all five sections). The Mauthner axons could not be traced farther caudally, because they are very close to the Müller fibres, and are at this time of the same calibre as these fibres. Most of the Müller axons are omitted, for clarity.

Only the anterior axon-collateral is shown in the figure. On one side, this process appears to be traceable into the opposite ventral motor column at about the level of entry of the maxillo-mandibular nerve; however, this collateral is slender and is close to an arcuate fibre crossing the mid-line. (Higher

magnifications could not be used because their field did not include suitab[le] reference-positions.)

The Mauthner dendrites are above the level of the otic capsules, but th[e] capsule ends dorsally at this stage in a vertical endolymphatic duct; th[is] enabled the two upper sections to be aligned with those below. The main den[-]drite continues the line of orientation of the cell-body, running dorsall[y,] laterally, and anteriorly towards the point of entry of the VIIIth nerve. Hor[i]zontal sections of older (stage 14) material show that this thick dendrite send[s] out short branches from its lateral aspect: most of these are directed laterall[y,] but a few point longitudinally; these are probably in contact with a colum[n] of Vth nerve fibres, as explained below.

This figure also shows that the Müller cells are taking up the positions an[d] the symmetry described by Stefanelli and others (compare fig. 1, p. 166).
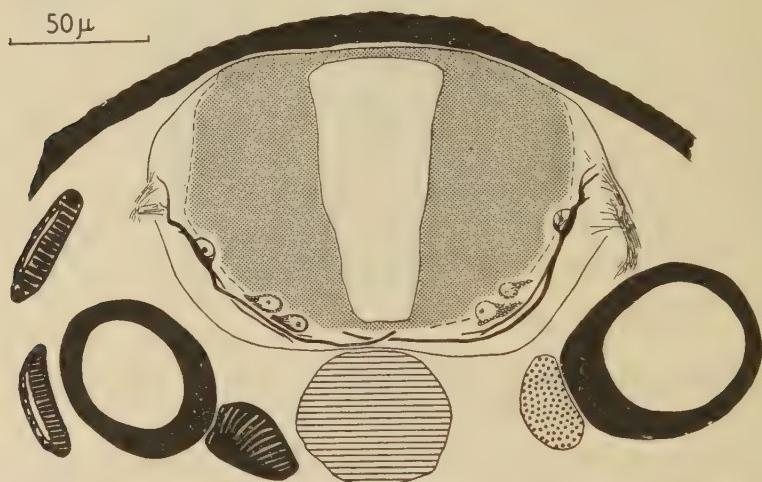
FIG. 5. From transverse sections of a *Lampetra* embryo (stage 11); the Mauthner neurones seen from behind, projected on to the level of the otic capsules and VIIIth nerves. Camera lucida drawing. Light stipple: grey matter. Heavy stipple: VIIIth nerve ganglion.

In fig. 5, the Mauthner neurones have been drawn from transverse sections cut at 8 μ, of a stage 11 embryo. The section on to which the neurones ar[e] projected passes through the outer parts of the fourth myotome on both sides through the front end of the inner part of this myotome on the left, and jus[t] anterior to the inner part on the right; this is the level of the upper part of th[e] Mauthner cell-body and the base of the main dendrite.

Mauthner dendrites extend, anterior to the section drawn, through on[e] section. The chiasma of the Mauthner axons lies in the same section as th[e] glossopharyngeal ganglion, eight sections posteriorly to that drawn. Below th[e] Mauthner cell-bodies, the position of the Müller cells nearest to them i[s] shown.

This figure confirms relations shown in figs. 2–4. The obliquely sited cell-body is dorsal to Müller cells lying along the somatic-motor and viscero-motor columns. The large main dendrite ascends dorsally to the position of entry of the VIIIth-nerve fibres, where its thickness makes it prominent in transverse sections: here three or four smaller dendritic branches extend laterally. These branches do not pass beyond the contours of the brain; no synapses are yet observable on them, but they are of course in close contiguity with incoming fibres running parallel with them. The animal is too young for all the separate, very complex, divisions of the lamprey's acoustico-lateral system to be safely distinguished (compare Kappers and others, 1936, p. 439–43), but these dendrites are probably contiguous with the vestibular division of the VIIIth nerve.

The axon and its branches have the course described for the preceding series. The lower branch of the axon-collateral ends rather abruptly, as shown: the upper branch continues as a slender process, towards the mid-line; its full extent could not be shown in the figure. The axon continues caudally beyond the chiasma, as a fibre of constant and prominent thickness.

Examination of pro-ammocoetes of stage 14, cut in orthodox planes, has given further information. In a transverse series, the Mauthner axons have been followed from the cell-body across the chiasma into the mid-lateral position in the cord, as far as the level of the anus, where this series stops. The graduation in calibre between the different longitudinal fibres is greater by this stage: tracing of individual fibres for long distances becomes practicable. When the Mauthner fibres have reached the mid-lateral position in the cord, in the innermost part of the white matter, they are easily distinguished from all the smaller longitudinal fibres near them (fig. 6, E). I have already illustrated them, and described their course in the spinal cord, without being aware of their identity (Whiting, 1948, figs. 10 and 11 and p. 374).

Parasagittal sections of a stage 14 animal (fig. 6, B) have also been drawn by camera lucida. This series confirms that the Mauthner fibres in the floor of the hind-brain are continuous caudally with the mid-lateral fibres in the spinal cord, across the gap shown in fig. 2.

The course of a Mauthner fibre along the spinal cord will bring it into contact with the *dendrites* of successive somatic-motor neurones down the trunk (fig. 6, C, D). There appear to be two types of somatic-motor neurones, primary and secondary: the primary motor neurones only reach the position of the Mauthner fibres through the dorsal end of the main dendrite, while the dendrites of the secondary system are much closer to them (Whiting 1948, 1955).

The Müller fibres, lying ventro-medially in the cord, will effect their co-ordinating function differently, either through funicular cells in the ventral part of the cord or directly upon the primary somatic motor neurones by way of the *ventro-medial process* of these neurones. This process is probably an axon-collateral rather than a dendrite; also, it often extends into close connexion with the contralateral motor column. It is, in any case, separate from

the main dendrite at this time and apparently remains so (compare Kappers and others, 1936, p. 153 and fig. 67, B).

The earliest identification of Mauthner neurones is in a stage 7 embryo, where the Mauthner neurones and one pair of Müller neurones can be seen. The cell-body is high on the side of the brain, the chiasma is visible, and the axon is beginning its descent of the spinal cord in a mid-lateral position. However, the Müller axons are also in a relatively lateral position at this time.

The Mauthner neurones are extremely constant in their relation to the rest of the nervous system. This can be clearly seen in fig. 7, A–C. Fig. 7, D, E amplifies some features of figs. 3–5: particularly the relatively large calibre of the Mauthner fibres at the chiasma, compared to other crossing fibres, and the isolated position of the Mauthner cell-bodies, dorsal to those of the Müller neurones.

CONCLUSIONS

These results show that the brain of the embryonic and early larval lamprey contains a pair of co-ordinating neurones having the main features of the Mauthner neurones found in fishes and amphibia: a very large cell-body lying in the hind-brain at the level of the otic capsule; a dendritic system in direct connexion with the VIIIth nerve; a very large axon crossing the floor of the hind-brain and forming a symmetrical chiasma with its fellow; this axon descends the contralateral side of the spinal cord.

No other pair is set apart in this way. No Müller neurone has a well-marked direct connexion with the VIIIth nerve yet, although we know from the work of Johnston, Pearson, and Stefanelli that cells 9 and *10* will develop this connexion later. No Müller neurones have axons descending the contralateral side of the cord: from stage 7 onwards, the axons descend the ipsilateral motor tracts, as so often described in older lampreys.

Axons, other than those of the Mauthner pair, which do cross the floor of the hind-brain, have not yet been traced to their full extent. All at present appear to be typical arcuate fibres, derived from cells dorsal to the Müller cells. The larger arcuate fibres are derived from levels of the brain too far above or below the otic capsule for the cell of origin to be in direct communication with the VIIIth nerve.

The interpretations made by previous workers will now be briefly stated, and assessed in the light of the results given above.

Johnston (1902) considered that a pair of Müller neurones lying 'in the lateral motor column at the level of VII' is 'possibly directly homologous with Mauthner's cells in *Acipenser*'.

FIG. 6 (plate). The C.N.S. of *Lampetra* larvae (stage 14). A, sections in similar plane to that of fig. 3: head to left. An arrow indicates the Mauthner cell and axon. B, parasagittal section of the hind-brain and spinal cord in an 8-mm larva. Mauthner fibre shows on right and again on extreme left. C, T.S., spinal cord showing a primary motor neurone. D, as C. The dorsal extent of the dendrite can be seen in both. E, T.S., anterior spinal cord, showing Müller fibres ventrally and the pair of Mauthner fibres at '3 o'clock' and '8.30'. F, T.S. of an 8-mm larva. Mauthner cell on right; two Müller cells and base of Mauthner dendrite, left. G, part of F, on larger scale.

25μ
A,B,C

50 μ
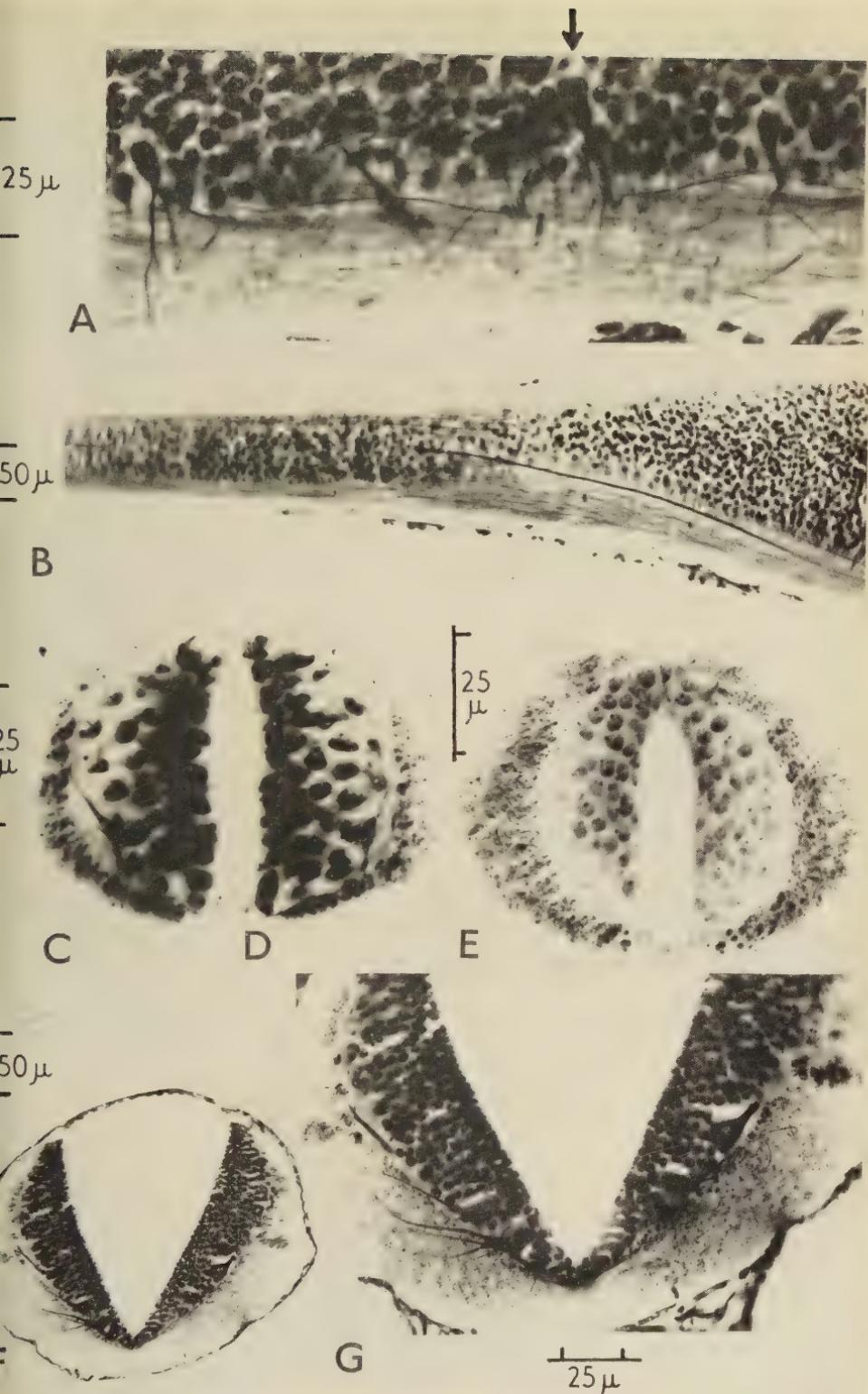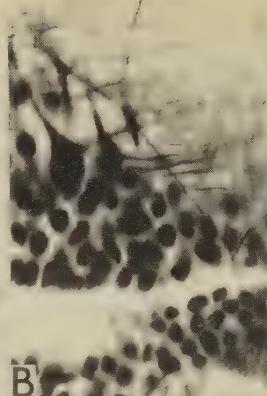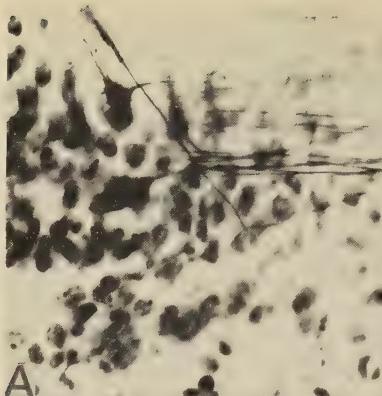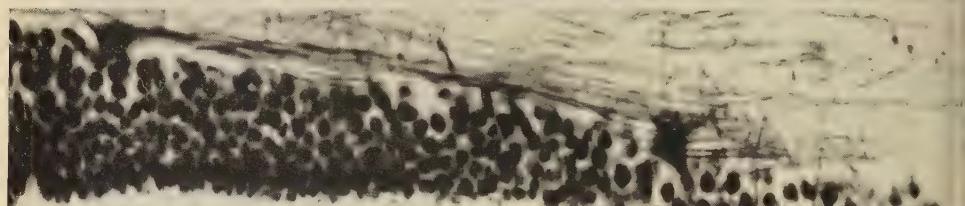
Fig. 7
H. P. WHITING

Stefanelli considers that the Müller pair 9 'is homologous, from its locations nd connexions, with the M(authner) cells, but its morphological charac-ristics are similar to those of other giant pairs' (Stefanelli, 1951). Stefanelli nd Camposano (1946) agree that the axon of cell 9 follows an ipsilateral ourse—'decorra lungo il midollo omolateralmente'—but compare these Iüller cells with the Mauthner cells of *Anguilla* and consider that the small ut crossing axons of *Anguilla* bridge the gap between normal Mauthner cells nd this Müller pair. Pair 9 is also illustrated by Addens (1933, fig. 59), Bar-ard (1936, fig. 2), and Woodburne (1936, fig. 3*h*): its identity is not in doubt nce it is described lying between the motor centres of the trigeminal and cial cranial nerves.

Pearson (1936), working partly on the same material as Barnard and Wood-urne, but studying different connexions, describes a Müller neurone dis-nct in position and connexions from that of Barnard and Woodburne. This ell lies at the same transverse level as the *ventral nucleus* (B, fig. 1), the chief estibular centre in the lamprey's acoustico-lateral area; it is also at the same vel as the incoming VIIIth nerve fibres, with which it is in direct synaptic elation. This is not cell 9, but is almost certainly cell 10. Comparison of earson's figure with that of Johnston (1902, fig. 11*a*) shows that this is the ell which Johnston considered as the Mauthner homologue, and Pearson also oncluded that the relations of this cell 'strongly suggest it is the forerunner of Iauthner's cell'. Stefanelli (1937) considered that Pearson's theory con-urred with his own. I think Stefanelli was mistaken: two distinct pairs of Iüller cells have been proposed as homologues of Mauthner cells.

Kappers and his colleagues (1936, p. 442) have put forward a third possi-ility. The forerunner of the Mauthner neurone may be one of the larger rcuate cells in the ventral nucleus in the acoustico-lateral area (this nucleus robably corresponds with *Deiters's nucleus* of higher vertebrates; compare eccari, 1943, and Cordier, 1954). These cells are concerned with vestibular eflexes and some of the axons cross the hind-brain to descend the contra-ateral side of the spinal cord in the medial longitudinal bundle: a hypothetical eurone *K*, having this relationship, is shown in fig. 1.

This possibility is strengthened by conditions in *Myxine* (Jansen, 1930), here some of the Müller-type neurones have a contralaterally running axon: nese cells are only distinguishable from arcuate cells by their size. But the coustico-lateral system is greatly reduced in hagfishes, so that a detailed com-arison of cells in this system with similar cells in other animals would be lappropriate.

FIG. 7 (plate). Mauthner neurones in horizontal sections of *Lampetra*.
A, B, C, sections of stages 12, 13, and 14 larvae, cut almost in horizontal plane; head to left. rom upper left of each picture, the Mauthner axon runs obliquely, across several Müller bres, to the chiasma. Neural canal and chordal cells can be seen in lower part.
D, stage 14 larva. On left, mid-brain-hind-brain *sulcus* in wall of neural canal: on right rrow), chiasma of Mauthner fibres. Two pairs of Müller cells can be seen.
E, the same embryo. A more dorsal section, aligned with D and to same scale, showing the air of Mauthner cell-bodies, just to the right of the scale-line.

Recent accounts of the vertebrate nervous system do not accept a homolog
between the Mauthner cell and any particular neurone of cyclostomes o
selachians. Beccari (1943, p. 187) contrasts the Müller cell with the Mauthne
cell, which 'differisce essenzialmente per il comportamento del neurite' an
emphasizes the special relation of the Mauthner neurone to the nucleus o
Deiters. The same two points appear from Cordier's diagram (1954, fig. 132
which includes both Müller and Mauthner neurones.

The validity of these theories may now be assessed. Although the fate of th
Mauthner neurone during ammocoete and adult life is still unknown, it nov
seems improbable that this cell can become transformed into either of th
Müller cells with which Stefanelli, Johnston, and Pearson wished to home
logize it. However, statements about this particular pair of neurones form onl
a minute part of the array of facts presented in these previous contribution
to our knowledge of the nervous system of cyclostomes.

On the other hand, the identification by Ahlborn of Mauthner fibres in hi
material must now be considered as probably correct. It is significant that h
was working on younger material than that studied by the later workers. It i
therefore possible that the Mauthner neurones have degenerated or becom
much less prominent, in the stages studied by Tretjakoff, Johnston, Pearson
and Stefanelli.

It is therefore reasonable to conclude that the pair of neurones which ha:
been termed the Mauthner pair in this account is homologous with the Mauth
ner neurones of fishes and Amphibia and should correctly be given this name

This in turn suggests that the whole mechanism by which the brain co:
ordinates the activity of the spinal cord, and so of the trunk-myotomes, mus
be homologous in the ichthyopsid vertebrates, up to at least the stage o
development when they can first swim, for the reasons given in the introduction

On the basis that this homology is accepted, a more detailed comparison
may be made. During further development from the 4-mm embryo shown in
fig. 5, the neural canal will become broader and shallower and the grey matter
will be rotated outwards and downwards about a centre at the median raphé
An intermediate stage in this process is shown in the 8-mm larva in fig. 6, F
and a final stage in a section of the adult brain at this level, e.g. figure 225 o:
Kappers and others (1936). Consequently the main dorsal dendrite of fig. 5
will be rotated so that it points laterally, while the axon will come to poin
more medially and less ventrally. These are the relations shown in most figure:
of gnathostome Mauthner cells, where older stages are usually being described
e.g. the Mauthner neurone of *Salmo* alevin larvae of Beccari (1943, fig. 145) o
Kappers and others (1936, fig. 194). The main or dorsal dendrite of the pro
ammocoete corresponds then with the main or *lateral dendrite* of typica
Mauthner cells.

In fishes and Amphibia, the lateral dendrite is in synaptic relation with vesti
bular fibres of the VIIIth nerve, and passes close above the 'descending column
('spinal V') of the Vth nerve, which runs longitudinally just below the entry
of the VIIIth nerve, carrying the somatic sensory component of the snout to

vagal and spinal levels. These relations of the Vth and VIIIth nerves are also found in lampreys (fig. 6, G and Woodburne's figures). The main dendrite of the pro-ammocoete Mauthner neurone sweeps over the 'descending V' column of fibres on its way to the VIIIth-nerve vestibular fibres, in just the same way.

In fishes and Amphibia, there is also a large *ventral dendrite* which runs ventrally among the many longitudinal columns of fibres, especially trigeminal and tecto-bulbar groups. This dendrite leaves the cell-body close to the axon-hillock. In younger stages, when the axon was directed more ventrally, for the reason given above, the axon and ventral dendrite would arise from a common stem, just as the axon and axon-collaterals do in the Mauthner cell of the pro-ammocoete. These axon-collaterals correspond in origin, course, and apparently in connexions, with the ventral dendrite of other ichthyopsids: the forwardly directed process from the axon-collateral, shown in fig. 4, is probably coming into contact with the tecto-bulbar system.

There is one striking difference between the Mauthner cells of the pro-ammocoete and those of jawed vertebrates: the position of the Mauthner fibres in the spinal cord. After the pro-ammocoete period, the spinal cord becomes flat and ribbon-like: in some species, two or three pairs of giant fibres then run in a more lateral position (compare Kükenthal, 1929, fig. 218). The lateral position of the Mauthner fibre in the pro-ammocoete may be linked with the approaching flattening of the cord—the growth-factors showing their effect upon this fibre first.

A similar change of position has been described in the hagfish *Myxine* by Jansen (1930). Some large co-ordinatory fibres, descending the hind-brain floor in the median ventral column, turn outwards and dorsally as they enter the cord, where they run with a motor column which is sufficiently lateral and dorsal in position to lie dorsal to the motor roots. This appears to be a closely parallel example.

Finally, the facts given here have supported the theory of Kappers and his colleagues in that the pro-ammocoete has a Mauthner neurone with the characteristics of their theoretical cell, termed here cell *K*. But this theory postulated that a unique and giant pair of cells has differentiated from among a number of smaller, similar cells, presumably at the phylogenetic juncture between Agnatha and Gnathostomata. The Mauthner cell was in fact developed at a much earlier phylogenetic stage.

It seems probable that a course of evolution may have occurred that is opposite to that proposed in their theory. The Mauthner neurone may be derived from a cell with an arcuate fibre, which developed its special Mauthnerian characteristics before there were any other neurones in the centre—'Deiters's nucleus'—in which it now lies: the Mauthner neurone would be a precursor or pioneer-neurone for the other cells in the same centre, not a derivative of them. Large and distinctive neurones are found more often among primitive chordates or younger stages than among advanced or adult ones. The ontogenetic and phylogenetic history of Rohon-Beard neurones, from the lamprey to man, is an example of evolution in this direction. The

Rohde cells of *Amphioxus*, cells A and B mentioned in this paper, and the Müllerian cells of cyclostomes, *Amiurus, Anguilla, Gymnarchus, Xenopus*, and *Tropidonotus* provide similar evidence. The ancestor of the vertebrate would possess numerous large and distinctive neurones, paired or unpaired, carrying out between them sensory, motor, correlating, and co-ordinating functions: such a team, measured in hundreds and not millions, might at first be the predominant part of the central nervous system, as it is still in the motile stages of lower vertebrate embryos.

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Observations on Photophores and Luminescence in the Teleost *Porichthys*

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With two plates (figs. 3 and 4)

SUMMARY

Porichthys myriaster is a shallow-water teleost possessing numerous photophores on head and trunk. These are embedded in the dermis and consist of a lens, layer of photogenic cells, and reflecting sheath. The photophore receives a nerve and blood-vessels which proceed to the photogenic tissue. The photogenic cells are finely granular and stain poorly. Luminescence in the living fish was evoked by electrical excitation and injection of adrenaline. When the nerve-cord was stimulated by electric shocks, the photophores lit up after a latent period of 7–10 sec. With localized stimulation a response was still obtained from all the photophores of the body after transection of the nerve-cord and arrest of circulation. Injection of adrenaline into the heart caused widespread luminescence in 2 min. The pattern of innervation of the photophores in teleosts suggests an autonomic supply. It is concluded that the photophores of *Porichthys* are probably innervated by the sympathetic system. Efferent fibres may be adrenergic. If the adrenals are involved, their effect is secondary, following nervous excitation.

INTRODUCTION

MOST species of teleosts having photophores are pelagic in habit, and occur in deeper waters. For this reason little is known about the functioning of their light organs (Harvey, 1952; Marshall, 1954). Myctophids, captured at the surface at night, are delicate, but can be observed for short periods in captivity. The photophores of myctophids emit transient flashes, the short latency and brief duration of which are indicative of nervous control (compare Beebe, 1926). There is a teleost, the 'midshipman' *Porichthys*, well endowed with photophores, that occurs inshore. The genus *Porichthys*, containing several species, is found off the west coast of America: it is common, large, and hardy, all of which factors make it favourable for physiological studies.

During a recent visit to California I was able to collect several specimens of *P. myriaster* Hubbs and Schultz at the Scripps Institution of Oceanography and the Kerckhoff Marine Laboratory. Some experiments were performed on the living animal in the limited time available, and material was prepared for histological study. The aim of this research was to discover whether the photophores of *Porichthys* are under nervous control.

Earlier work by the Greenes showed that the photophores of *Porichthys* could be caused to luminesce by electrical stimulation of the whole fish, and by injecting adrenaline or pituitrin. Since nerve-fibres to the photophores

were little in evidence, it was concluded that regulation of luminescence was hormonal (Greene, 1899; Greene and Greene, 1924).

ARRANGEMENT OF THE PHOTOPHORES

The various species of the genus *Porichthys* differ slightly in the distribution of photophores, but the general pattern is similar. A detailed study of the photophores of *Porichthys* was carried out by Greene, who examined several species, including *P. myriaster* (Greene, 1899; Hubbs and Schultz, 1939).
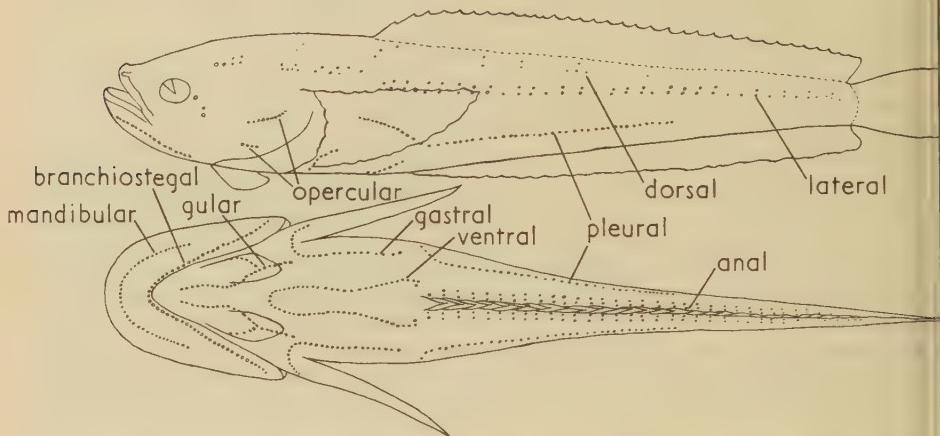
FIG. 1. Diagrams showing the disposition of photophores in *Porichthys*. (After Greene, 1899.)

Porichthys possesses four lateral lines and the photophores are disposed in rows which generally accompany the multiple lateral lines. For brevity of description the photophores can be separated into a cephalic series and a trunk series (the latter including those on the throat). The photophores on the head lie in branchiostegal, mandibular, and opercular rows. Those on the trunk include lateral, scapular, dorsal, dorsal accessory, pleural, anal, gastral, and ventral rows. A gular row occurs on the throat. The location of the light organs is shown diagrammatically in fig. 1, adapted from Greene (1899).

EXPERIMENTAL OBSERVATIONS

Seven specimens of *P. myriaster* were available for experimental work. The animals varied in weight from 0·5 to 1·5 kg. Only some of the experiments were successful, but experience was otherwise gained in handling the material. The fish were anaesthetized by intraperitoneal injections, at first of veronal, which was weak in action; later, of nembutal. While under anaesthesia, the fish were moistened with sea-water, and running sea-water was passed over the gills. Operated areas were swabbed with *Lophius*—Ringer (Young, 1933). Nervous stimulation was carried out by means of condenser discharges (4 μF) from an electronic stimulator. The electrodes were a pair of closely spaced bare silver wires. These were placed on the medial dorsal surface of the spinal

cord, well above the somata of pre-ganglionic visceral neurones. Voltages at the electrodes varied from 10 to 60 V; true stimulating voltages were of course less, owing to leakage of current through saline and intervening tissue. Room temperatures were 21–24° C; the temperature of running sea-water varied from 17 to 18° C.

Effect of electrical stimulation

Four animals were used for electrical stimulation. The spinal cord was exposed by dorsal approach at a level corresponding to the anterior edge of the pectoral fins. Much difficulty was encountered by the rapidity with which blood and tissue exudate coagulated, forming a firm gel over the nerve-cord.

When the spinal cord was stimulated with bursts of shocks, above threshold, the photophores lighted up slowly. Varying the conditions of stimulation established the following relationships.

An increase in voltage, above threshold, caused the response to become brighter. Thus, with bursts of pulses (3 per sec for 1 min), the lateral photophores shone more brightly when the stimulus-strength was raised from 44 V to 60 V.

A brighter response was obtained by increasing the number of shocks. Bursts of shocks (same strength) at 3 per sec were administered for various periods:

2-sec burst produced very faint light (doubtful).
5-sec burst evoked faint light in the lateral photophores.
10-sec burst caused the photophores to glow more brightly.

A prolonged burst for 1 min at 3 per sec produced a bright response in which all the photophores lighted up, i.e. those in lateral, ventral, and cephalic rows. A definite impression was obtained that the photophores of the trunk lighted up first, and that the response then spread swiftly anteriorly, to invade the photophores of the head.

Stimulation at frequencies below 3 per sec was ineffective, or produced a very weak response. Thus, in one animal, a burst lasting 1·5 min at 144 per min failed to produce luminescence, but when the frequency was switched to 4 per sec, light quickly appeared in the photophores. Interestingly enough, blanching (chromatophore contraction) was induced at a lower frequency (3 per sec) than luminescence. To cause the photophores to light up in this animal a burst lasting 15 sec at 4 per sec was required. Effective stimulation at this level induced luminescence in both head and trunk, light in the cephalic photophores coming up later and appearing weaker than in those of the trunk. Raising the frequency to 10 per sec induced a much brighter response.

Temporal characteristics of the response showed some variation. With a 5-sec burst at 3 per sec a feeble response (faint light) appeared in 15 sec. With a burst lasting 10 sec at 3 per sec, light appeared in 7 to 10 sec. The glow rose to a maximum in 1 min and gradually faded after another 1·5 min.

In two animals the spinal cord was transected at the level of the pectoral fins and stimulation was repeated. The electrodes were placed on the cord

behind the cut. In one animal a burst of shocks at 3 per sec for 2 min elicited
a bright luminescent response: all the photophores lighted up, including those
on the trunk, throat, and head. In the other specimen a burst at 10 per sec
induced bright luminescence in the photophores posterior to the cut; the
response then extended to the photophores of the head.

When the cord was stimulated anteriorly to the cut at frequencies from
3 to 10 per sec, all the photophores lighted up. The response appeared at first
in the photophores of the trunk behind the cut, and extended posterio-
anteriorly to invade the photophores of the head.

An attempt to evoke luminescence by stimulating the cord in the anterior
caudal region was unsuccessful.

In order to eliminate any possible hormonal effect in these experiments,
the circulation was stopped by ligaturing the heart at the bulbus. The spinal
cord was then stimulated by a 2-min burst at 3 per sec. All the photophores
lighted up, both those of head and trunk.

Effect of injecting hormonal preparations

Following up the earlier work of the Greenes (1924), I have sought to
induce luminescence in *Porichthys* by injecting adrenaline and two pituitary
preparations.

A fish weighing 1·03 kg was anaesthetized and the heart exposed. A solution
of adrenaline was injected directly into the ventricle. The dosage was 0·1 ml
of 1/100 adrenaline in isotonic saline (Burroughs Wellcome epinephrine).
The adrenaline caused all the photophores to light up, the effect appearing
2 min after injection.

A fish weighing 0·5 kg was used to test pituitrin. It was injected intra-
abdominally with 1 ml of saline, without inducing luminescence. After this
treatment it was injected intra-abdominally with 2 ml of a pituitrin solution in
isotonic saline (dosage equivalent to 6 I.U. of Parke, Davis & Co. obstetrical
pituitrin solution). This solution did not evoke luminescence in 30 min.

Two specimens, weighing 1 and 1·5 kg, were injected intraperitoneally
with purified melanophore hormone (Armour). The dosage per fish was 10 mg
hormone in 5 ml of half-strength sea-water. Ten minutes after the injection
four photophores on the ventral surface of one fish lighted up and remained
glowing over the course of the next hour. No other photophores lighted up in
this fish, and no luminescence appeared in the other animal. The photophores
which responded were over the area of injection, and mechanical stimulation
or injury may have been involved in the responses.

HISTOLOGY OF THE PHOTOPHORES

For microscopic examination, pieces of skin containing photophores were
immersed in standard fixatives (Heidenhain's Susa, Helly's, Flemming's,
Bouin's, formaldehyde, picro-formaldehyde, 80% alcohol). The material was
sectioned in wax. Stains employed were: Masson's trichrome, Heidenhain's
Azan, haematoxylin eosin, and Bodian's activated protargol.

As described by Greene (1899), the photophores of *Porichthys* lie in the dermis. Each consists of a lens, beneath which is a layer of photogenic cells. Surrounding the whole is a sheath (figs. 2 and 4).

The lens is separated from the epidermis by the dermis, consisting of densely arranged connective tissue-fibres. The lens is formed of cells which tend to be flattened around the periphery, irregularly polygonal in the centre.
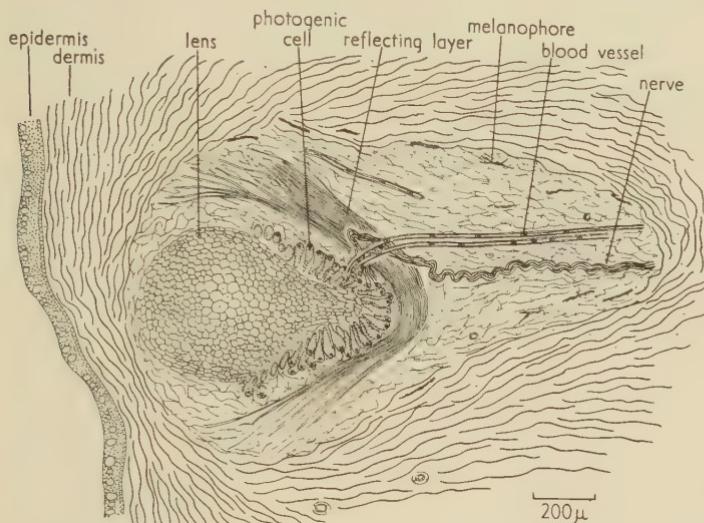
FIG. 2. A photophore of *Porichthys*, showing nerve and blood-supply. Camera lucida drawing from one section, with some details added from adjacent sections.

The cells are nucleated and have a homogeneous cytoplasmic content. They stain deeply with eosin and xylidine red. When treated with Masson's trichrome, the central cells become deeply blackened with iron haematoxylin, whereas the peripheral cells are red. With Azan the cells are stained deeply by azocarmine and also take up some aniline blue, so that they appear reddish-purple. They are impregnated uniformly with silver, after which they are ringed with black margins. In places where the cells are slightly separated from one another, dentate bridges can be seen linking them together. The black margins brought out by silver represent intercellular cement.

The photogenic tissue consists of a single epithelial layer in which the cells are directed inwards from the periphery of the photophore towards the base of the lens (figs. 2; 4, B). The photogenic cell is broad basally and tapers somewhat distally towards the lens; in cross-section it appears circular. The nucleus of the photogenic cell lies towards the base. The cytoplasm is finely granular and stains very poorly. It is resistant to eosin, becomes at most a faint lavender colour after Azan, but is strongly coloured by the xylidine red of Masson's trichrome stain. The photogenic cells are coloured deeply after treatment with silver. Fine connective tissue-fibres (staining with aniline blue and light

green) extend into the photogenic layer from the immediately surrounding
sheath. These fibres form thin investing coats about the photogenic cells.

The connective tissue sheath extends downwards and inwards from the
dermis, and is continuous around the sides of the lens and the photogenic
layer, except for perforations for blood-vessels. The sheath consists of a
mesh-work of fine collagenous fibres staining with aniline blue and light
green. The interstices of the sheath are packed with fine crystals. These are
thin polygonal wafers having their flat surfaces parallel to the surface of the
sheath. By transmitted light the sheath appears dense and yellow; in reflected
light the sheath glistens brightly with a white or bluish hue (fig. 3). The
crystals of the sheath are dissolved in acid fixatives. The sheath forms a
reflector for light originating in the photophore.

Reflecting material of this sort is often regarded as guanine. The reflecting
particles in the photophore sheath show some of the solubility characteristics
of guanin: they are dissolved in 1% HCl, 1% NaOH, and 5% oxalic acid;
they are not affected by water, ethanol, xylene, ether, 10% acetic acid, or
10% NH_4OH. The sheath differs from true guanine, however, in being
insoluble in iron alum, saturated picric acid, and piperazine. Further, it shows
no selective blackening with ammoniacal silver nitrate (Millot, 1923; Lison,
1936).

Outside the reflecting tunic is a loose reticular layer which forms a sort of
loose bag about the photophore. It contains fine fibrils that stain lightly with
aniline blue and light green, and it is traversed by nerves and blood-vessels
extending from the dermis proper to the photophore (fig. 2). Blood-vessels
enter the base of the photophore through the reflector layer, and supply the
photogenic tissue.

A few scattered pigment cells (melanophores) lie in the loose connective
tissue-bag surrounding the reflector layer.

The nerve-supply for the photophore consists of a bundle of fibres which
approach the photophore laterally from the dermis. The nerve passes through
or over the top of the reflecting layer, and its fibres extend into the photogenic
tissue (figs. 2; 4, C).

Measurements made on a typical photophore, having a width of 660 μ, are
as follows (fig. 5):

thickness of overlying epidermis, 100 μ; of dermis, 275 μ;
diameter of lens (perpendicular to the epidermis), 250 μ;
thickness of photogenic layer (in central vertical axis of the photophore),
    75 μ;
width of photophore proper (that portion bounded by reflecting layer),
    330 μ;
cellular dimensions: lens cells, up to 25 μ; photogenic cells, 15 μ by 32 μ.
This photophore contained about 600 to 700 photogenic cells.

FIG. 3 (plate). Views of the sheath or reflector layer of the photophore by reflected light.
A, section cut longitudinally through the sheath in the centre of the photophore.
B, longitudinal section through the margin of the sheath.

FIG. 3

J. A. C. NICOL

loose reticular tissue

reflecting sheath

photogenic tissue

epidermi[s]

dermis

lens

200 μ

A

lens

photogenic cells

sheath

50 μ

B

nerve

reflecting layer

100μ

C
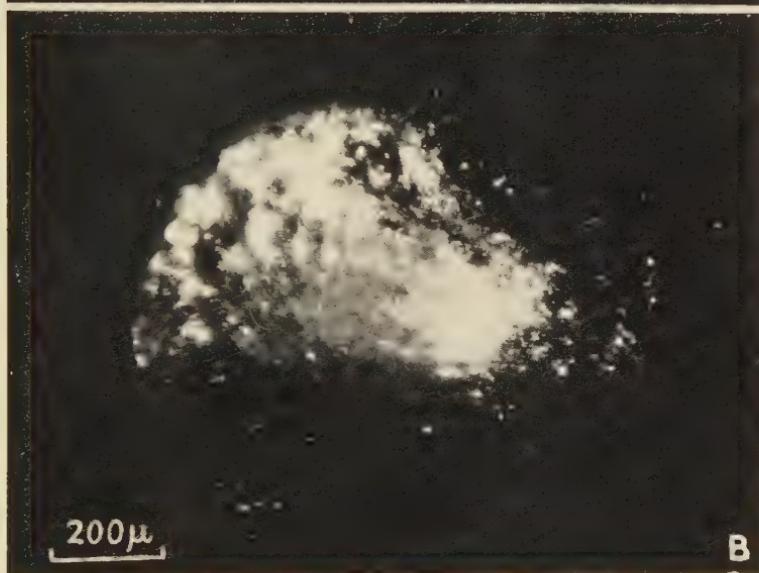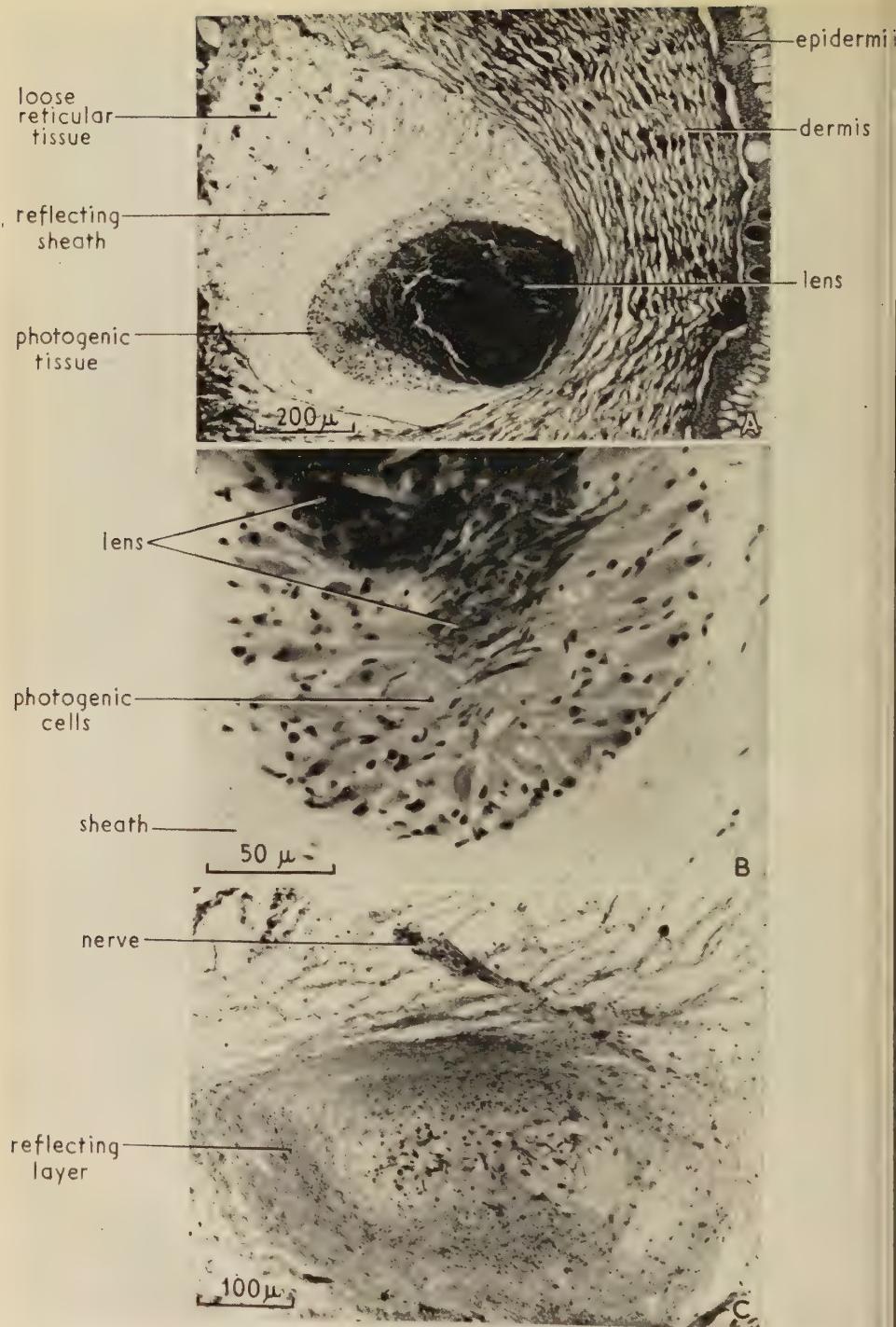
Fig. 4

J. A. C. NICOL
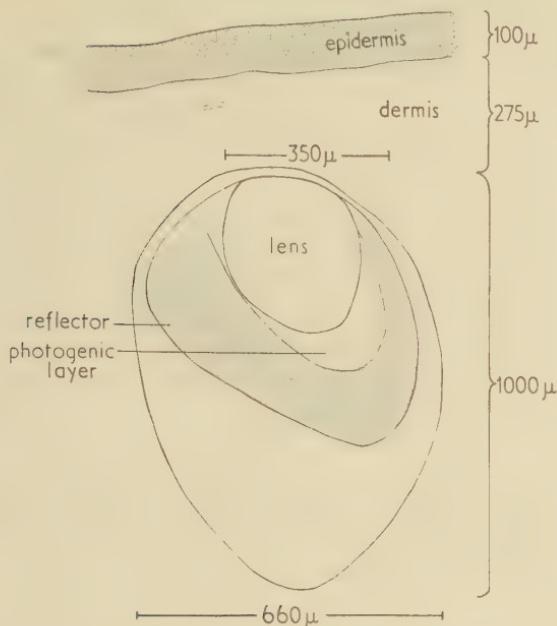
FIG. 5. Diagram of a photophore (from a camera lucida drawing).

DISCUSSION

Greene (1899) believed that the photophores of *Porichthys* were not in-
ervated, or at least lacked a special set of efferent nerve-fibres. Occasional
bres seen entering the light organs were considered to be branches of the
eneral cutaneous supply (presumably sensory fibres to the skin). He found
at faradic stimulation of the fish did excite general luminescence, and he
scribed this result to direct electrical stimulation of the photophores. Having
iscovered that injection of adrenaline induces luminescence, Greene and
reene (1924) concluded that the photophores were under hormonal control.

The present results show clearly, however, that luminescence is under
ervous control. It is evoked by direct and localized stimulation of the spinal
ord. Although Greene (1899) believed that he was exciting the photophores
irectly, it is significant that he speaks of placing one electrode over the
rain: it is more likely that he was stimulating the central nervous system
ith rapid and strong induced shocks. The effects of varying the conditions
f stimulation are suggestive of nervous control. The brightness of the re-
ponse, and sometimes its spatial distribution, are increased by increasing the
equency and number of shocks, or the stimulation-voltage. These effects
ay well depend upon recruitment of additional efferent neurones, and upon

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FIG. 4 (plate). Photophores of *Porichthys*.
A, general view (Helly, Azan).
B, photogenic tissue (formaldehyde, Masson's trichrome).
C, innervation (Helly, Bodian's silver).

summation or facilitatory influences engendered by repeated impulses in the efferent pathways, or in the photocytes.

The rapid onset of luminescence, in 7 to 10 sec after stimulation of the nerve-cord, also argues for nervous control. Similarly, the Greenes (1924) observed a latency of 8 to 10 sec for the luminescent response after electrical stimulation. These workers also discovered that adrenaline induced luminescence in the intact animal after a rather long latency. The first light appeared in 30 to 110 sec after intramuscular injection. Even when adrenaline is injected into the heart in fairly high concentration (1 mg adrenaline per kg of body-weight), the response takes 2 min to appear. The effective therapeutic level in mammals is only about 1 μg per kg of body-weight. The resting output of catechol amines from the adrenal glands is about 0·15 μg per kg per min, and is increased by about 40% during carotid occlusion (Kaindl and von Euler, 1951).

Adrenal (suprarenal) glands are present in the dorsal abdominal wall of teleosts (review by Nicol, 1952). If luminescence is dependent upon release of adrenaline into the blood-stream, the latent period of the response would involve, among other things, transmission time, secretion time, circulation time, and photophore latency. These parameters are largely unknown. Mott (1950) gives an estimate of 60 sec ($\pm$36 sec) for blood from the ventral aorta to reach the posterior cardinal vein in the eel. The prolonged latency observed after injection of adrenaline is in agreement with a long circulation time. It is unlikely, therefore, that the suprarenal glands can be involved in initiating the luminescent response under nervous stimulation. It has been shown, furthermore, that luminescence can still be evoked when the circulation is stopped. This proves that release and transport of a blood-borne hormone is not necessary for the response to appear. Although these results show that the nervous system alone can bring out all the features of a luminescent response, they do not preclude the possibility that, in the intact animal, hormonal control may be a contributory factor along with direct nervous regulation.

No unequivocal evidence was obtained to support the observation of the Greenes (1924) that pituitary extract induces luminescence. Apparently they made only one injection of pituitrin into a single specimen, and a clear positive response was obtained.

The discovery, in histological preparations, that nerve-fibres enter the photophores, provides confirmatory evidence for nervous control. Greene (1899) also notes several instances in which nerve-fibres were seen to reach the photophores.

Although nervous control of the photophores of *Porichthys* seems reasonably certain, there is no direct evidence as to the nervous pathways concerned. The innervation of photophores has been described in other teleosts, namely *Argyropelecus*, *Cyclothone*, and *Lampanyctus*. In these forms all the photophores of the head are innervated by the facial nerve (mandibular, buccal, and hyoid branches). A branch of the superior maxillary (trigeminal), supplying the preorbital light organ of *Argyropelecus*, would appear to include facial

fibres. In the trunk (as described in *Lampanyctus*), the photophores are innervated by ventral or medial branches of spinal nerves (Handrick, 1901; Ray, 1950). Neither of these workers traced the neuronal pathways involved, but from the pattern of distribution of efferent nerves, I hazarded the suggestion that they might be autonomic (Nicol, 1952).

The autonomic pathways of teleosts were worked out in detail by Young (1931) from *Uranoscopus*, a fish not very unlike *Porichthys*. In these animals the well-defined sympathetic trunks are connected with the spinal cord in each segment by white and grey rami communicantes, and there is a cephalic sympathetic extension which reaches the cranial nerves (III, V, VII, IX, and X). These provide avenues for the peripheral distribution of sympathetic fibres in the head.

It has been shown that the luminescent response still appears in normal pattern in the head and trunk after spinal section, i.e. it extends to all regions lying anterior to the section when the cord is stimulated behind the cut, and conversely. This result is strongly indicative of sympathetic control. The only longitudinal pathway still available after cutting the cord, apart from the lateral branch of the vagus, is the sympathetic system. By assuming that photophore-innervation in *Porichthys* is similar to that worked out for other teleosts previously mentioned, we can form a picture of the photophores of the head receiving their sympathetic fibres through the facial, and those of the trunk receiving sympathetic fibres which traverse recurrent grey rami and spinal nerves in each segment. The fact that the photophores are excited by adrenaline is compatible with sympathetic innervation, since this is a normal chemical transmitter of sympathetic fibres, and the photophore nerves may well be adrenergic in nature. Since suprarenal tissue is well represented in teleosts, the possibility exists that adrenaline secretion into the bloodstream is a contributory factor in a prolonged response. Other teleosts in which luminescence has been induced by injection of adrenaline are *Echiostoma ctenobarba*, *Argyropelecus olfersi*, and *Macrourolicus pennanti* (Harvey, 1931; Bertelsen and Grøntved, 1949).

Nothing is known about the ethological significance of luminescence in *Porichthys*. It is rarely evoked by tactile stimulation, and has not been observed to appear spontaneously (Greene, 1899). In myctophids luminescence is induced by tactile and visual stimuli. *Porichthys* lives well in captivity, and would appear to be a profitable animal for further studies of luminescence.

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Cone Arrangements in Teleost Retinae

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With two plates (figs. 1 and 6)

Summary

1. There are four types of cone elements in teleosts: single, double, triple, and quadruple cones. The latter two types have only been found as typical elements in minnow (*Phoxinus laevis*) retinae. The constituent parts of a multiple cone may differ from each other in staining properties and size.

2. A regular arrangement of single and double cones is a feature of many teleost retinae, but these cone patterns are only associated with equal double cones.

3. Changes in the cone patterns occur during growth of the retinae. In trout (*Salmo trutta*) the pattern in young eyes has many more single cones than that in adult retinae. The loss of these single cones is probably due to their transmutation into rods.

4. The derivation of the typical cone arrangement in central regions of the retina from that found at the periphery has been studied in trout, minnow, and pike (*Esox lucius*). In all these species there is a similar basic cone pattern at the edge of the retina, although the arrangements in more central parts are very different. It appears that the triple and quadruple cones in minnow retinae are formed by the fusion of a single cone with a double cone and a triple cone respectively.

Contents

Introduction

MOST teleost retinae contain single and double cones and these are frequently arranged to form a regular cone mosaic. Teleost cone patterns have been observed by a number of investigators, including Hannover (1840), Beer (1894), Eigenmann and Shafer (1900), Fürst (1904), McEwan (1938), Müller (1951), Ryder (1895), and Shafer (1900), but very few of them have studied the pattern in detail. The most comprehensive study is that made by Eigenmann and Shafer who enumerated seven different patterns and also claimed that the pattern was constant for a particular species. My observations on the growth of the trout (*Salmo trutta*) retina have shown that in this species the pattern changes as the eye grows (Lyall, 1957). Since the teleost retina grows from the edge, the differences between the central and peripheral cone arrangements, which have sometimes been observed, may also be

growth changes. The results of an examination of the retinae of various teleosts, with particular reference to the types of cones and their arrangements, are presented in this paper. The origin and possible significance of cone patterns and double cones, and the evolution of double cones, are discussed in relation to these observations.

METHODS

The retinae of various teleost species have been examined. Bouin and formaldehyde have been used as fixatives, and serial sections have been prepared in the manner described in an earlier paper (Lyall, 1957).

RESULTS

Types of cones

Single cones are the basic type of photopic visual cell found throughout the vertebrates. A teleost single cone consists of a conical outer segment and a cylindrical inner segment containing an ellipsoid. The nucleus is at the base of the inner segment and the cell terminates in a tapering foot-piece which passes through the layer of rod nuclei. In trout retinae I have occasionally found an unusual type of large single cones which, from their size and position in the cone pattern, appear to each represent half a double cone. *Double cones* are found in many vertebrate retinae and generally consist of two dissimilar halves fused together, one half resembling a single cone whereas the other half is larger and non-migratory. Teleost double cones differ from those of other vertebrates in that both halves undergo photomechanical movements and are usually of the same shape and size. These typical teleost double cones are generally called twin cones which by definition consist of two identical halves fused along their inner segments. The two halves of a trout double cone are of equal size and these elements are usually referred to as twin cones, but I have found that the two halves often stain differently with haematoxylin or Mallory and are therefore not identical. Similar staining differences were observed by Müller (1951) in *Lebistes* with azan stain, and Schultze (1867) observed that in some teleosts there was a difference in the appearance of the two halves of a double cone in a fresh retina, the cytoplasm of one half being more homogeneous than that of the other. According to Müller (1954) the staining properties of the cones (of *Lebistes*) change during dark adaptation. The majority of my sections have been taken from fully light-adapted eyes, but the differential staining of the two halves of the double cones is still evident in dark-adapted trout retinae. Several types of double elements in which one half differs morphologically from the other, to some extent, have also been observed in teleosts (e.g. by Butcher, 1938; Verrier, 1928; Walls, 1942), and these have been termed conjugate elements or unequal twin cones. The differential staining of teleost double cones with two halves of equal size indicates that the physico-chemical make-up of the two halves is not identical, and it is therefore debatable whether such structures should be referred to as twin

triple

quadruple

double

double

single

single
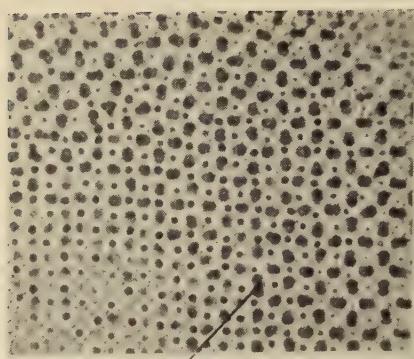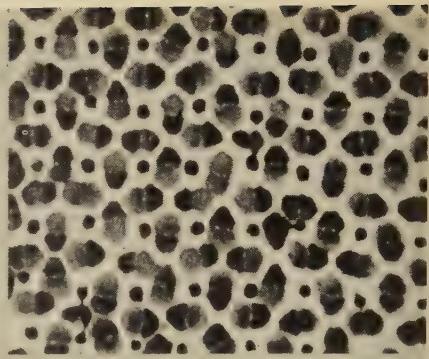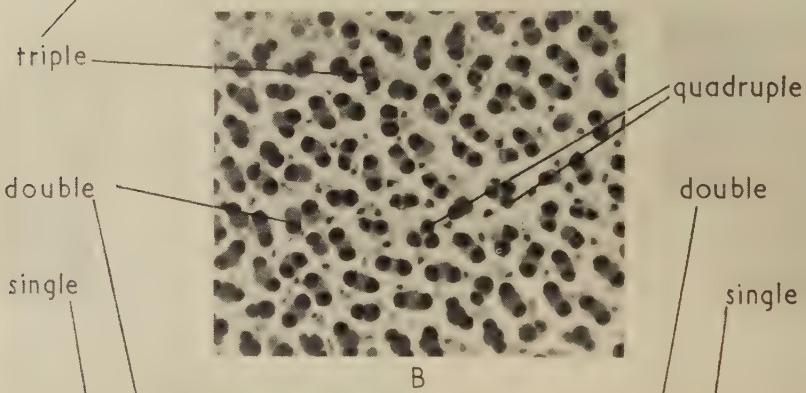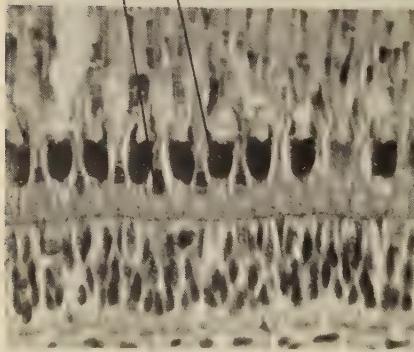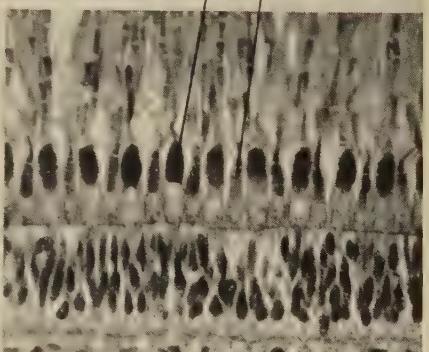
FIG. 1

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cones or conjugate elements. It is simpler to refer to all these double elements as double cones, irrespective of the extent by which the two halves differ. Double cones can be subdivided, on the criterion of size only, into equal and unequal double cones, to distinguish the unique type of teleost double element which has two halves of equal size from the unequal double cones of some teleosts and other vertebrates.

Triple cones have previously been observed as rare and anomalous structures; Saxen (1953) observed a number of triple cones in tadpole (*Rana temporaria*) retinae and Underwood (1951) found a few in gecko (*Aristelliger praesensis*) retinae. I have observed more than 150 triple cones, in which the three parts are of equal size and arranged linearly, in tangential sections of trout retinae (fig. 1, A). The position of the triple cones in relation to the general cone pattern indicates that these are abnormal double cones. In minnow (*Phoxinus laevis*) retinae triple cones are numerous and must be a fundamental type of visual element in this species. The three parts of a minnow triple cone are arranged linearly, as in trout, but the central cone is larger than the cones on each side of it. In longitudinal section the outer segments of the lateral cones are level with the ellipsoid of the large central cone. *Quadruple cones* consisting of three smaller cones arranged symmetrically around a large central cone are also present in minnow retinae. These quadruple cones are fairly numerous and in a few sections they are almost as abundant as triple cones. Each part of a multiple cone has the same structure as a single cone. Fig. 1, B shows double, triple, and quadruple cones in a tangential section of a minnow retina.

Cone patterns

The cone patterns are seen most clearly in tangential sections of the retina cut through the cone inner segments, but they can usually still be traced at the level of the cone nuclei. One of the most common patterns is that found in an adult trout retina (pattern I) (figs. 1, C; 2). Each pattern unit consists of four double cones surrounding a single cone; the double cones are arranged in two pairs so that the cones of each pair are parallel to each other and at right angles to the other pair. I have found a slightly different pattern (pattern II) in young trout retinae in which there is an additional single cone at each corner of a pattern I unit. Pattern II is shown in figs. 1, A and 3. The additional single cones are slightly shorter (33% or less) than the central single cones. The double cones are the longest and, contrary to Müller's (1951) observations in *Lebistes*, each half of a double cone has a greater diameter than either type of single cone. A regular alternation of double and

FIG. 1 (plate). A, tangential section of a young trout retina showing the cone pattern.

B, tangential section of a minnow retina.

C, tangential section of an adult trout retina showing the staining pattern.

D, longitudinal section of a trout retina showing the double cones cut through their long diameters.

E, longitudinal section of the same retina showing the double cones cut through their short diameters.

single cones is also evident in longitudinal sections when the plane of sectioning
coincides with a row of cones, and the single cones can be identified as central
single cones or additional single cones according to the plane in which the
double cones are sectioned (see fig. 1, D, E). The pattern in trout (which is
the one studied in greatest detail) is not regular over the whole retina, for the
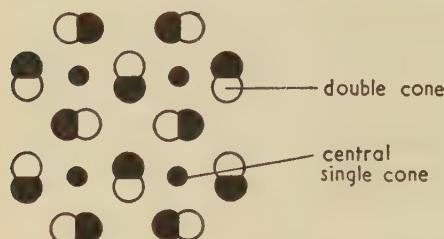
FIG. 2. Diagram of the adult trout cone
pattern and the staining pattern.
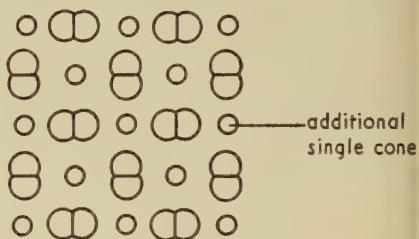
FIG. 3. Diagram of the young trout cone
pattern.

direction of the lines of cones sometimes changes and there are also irregu-
larities caused by the addition and termination of cone rows. Occasionally a
double cone is represented by a large single cone or by a triple cone (fig. 4),
but the central single cones are always present.

TABLE I

Teleost cone patterns

Genus	Pattern	Observer	Genus	Pattern	Observer
Perca		*	*Salmo*		*, 2, 3, 4, 7
Micropterus		8	,,		*, 4
Scorpaena		1	*Thymallus*		*
,,		2	*Esox*		*, 2
Cottus		*	*Barbus*		5
Blennius		1, 2	*Phoxinus*		6
Gasterosteus		6	,,	None	*
Gadus		*	*Lebistes*		6

* Lyall (1957).
1 Beer (1894).
2 Eigenmann and Shafer (1900).
3 Franz (1913).
4 Fürst (1904).

5 McEwan (1938).
6 Müller (1951).
7 Ryder (1895).
8 Shafer (1900).

Some teleost genera in which cone patterns have been observed are shown
in table 1 with their respective patterns. In perch (*Perca fluviatilis*) and miller's

humb (*Cottus gobio*) the single and double cones are arranged as in adult rout (fig. 2), but in the former species I have observed some variation in the ize of the cones in certain regions. I have observed both patterns I and II in
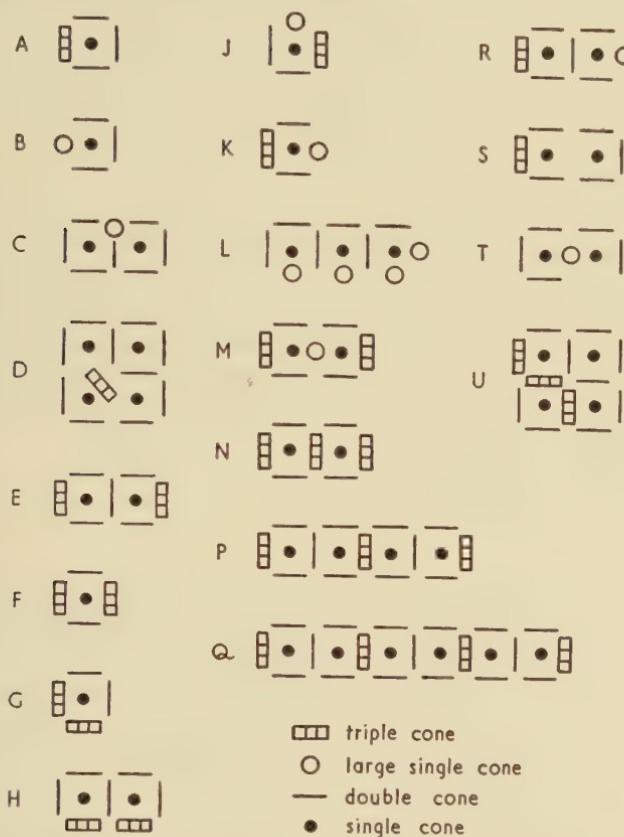
FIG. 4. Diagram of the variations in the trout cone pattern due to triple cones and large single cones. No additional single cones are shown.

lmon (*Salmo salar*) retinae. The cone pattern in grayling (*Thymallus vulris*) is similar to that in adult trout. The cone arrangements in char (*Salvenus willughbii*) and gwyniad (*Coregonus pennantii*) are rather irregular; the ne elements are chiefly double cones which are arranged nearly parallel to ch other over much of the retina. In some regions pattern I is visible, but e single cones are small and cannot always be distinguished in sections cut rough the inner segments of the double cones. The pattern in pike (*Esox cius*) is unusual in that the pattern units are triangular, instead of the more mmon rectangular units of patterns I and II. There is some difference of inion on the patterns found in certain species, e.g. that figured by Eigenann and Shafer (1900) for *Scorpaena porcus* differs from that given by Beer 894). Müller (1952) describes a cone pattern in *Phoxinus laevis* whereas I

have found an irregular arrangement of single, double, triple, and quadruple cones in this species. In central regions of minnow retinae I have only observed single cones in tangential sections cut near the nuclear layer; these cones are shorter than the other cones and also have a greater diameter than the constituent elements of the multiple cones. I have always found that cone patterns are associated with double cones in which the two halves are of equal size, although the retina of carp (*Cyprinus carpio*) has no pattern despite the presence of equal double cones. There is no pattern in the retinae of roach (*Rutilus rutilus*) and rudd (*Scardinius erythrophthalmus*) which have unequal double cones.

There is a regular arrangement of the differently stained halves of trout double cones, so that within the anatomical mosaic of single and double cones there is a staining pattern (figs. 1, C; 2). With haematoxylin, one half of a double cone stains darkly while the other half is eosinophil; a similar staining pattern is observed with Mallory. The double cones are arranged so that two light and two dark halves face each other alternately along each row and where the rows intersect two dark halves traverse two light ones. This regular staining pattern is not usually visible in young trout retinae and not in all the retinae of adult fish. The staining pattern in trout is the same as that found by Müller (1951) in *Lebistes*.

Changes in cone patterns

(*a*) *Changes in the central region of the retina.* Eigenmann and Shafer (1900) claimed that the cone arrangement in the retinae of teleosts was constant for a particular species and Müller (1951) confirmed this when he found the same pattern in the retinae of *Lebistes* of different ages. In trout and salmon, however, the pattern differs in young and adult fish, and many of the single cones present in the young trout retinae are absent from the cone pattern in adult trout. The loss of the additional single cones in trout, which occurs during the change-over from pattern II to pattern I, takes place gradually, so that in some regions a few pattern II units are scattered among those of pattern I. The change to pattern I involves the transformation of a retina in which the single cones and double cones were approximately equal in number to one in which the ratio of single to double cones is approximately 1:2. The loss of the single cones begins near the pole of the eye and progresses towards the periphery, and thus follows the course of earlier differentiation and development. The change-over occurs in fish 1–2 years old, so the loss of the additional single cones cannot be compared with the waves of degeneration which Glucksmann (1940) found in the retinae of frogs during differentiation. There is no evidence of the degeneration of the additional single cones and it seems likely that they are transmuted into rods (see p. 197). Fürst (1904), who observed the change of pattern in salmon, suggested that the missing cones might be found among the rods in the adult retina.

(*b*) *Changes between the periphery and the centre of the retina.* I have examined the peripheral cone arrangements in trout, minnow, and pike retinae and,
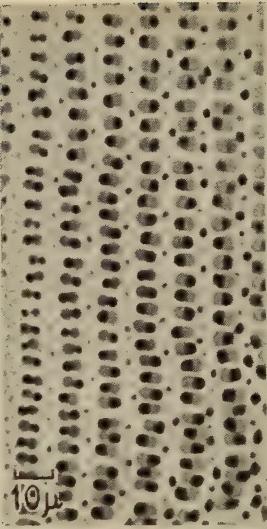
FIG. 6

A. H. LYALL

lthough they have very different cone arrangements in the centre of the retina,
here is a similar basic arrangement at the periphery. The retina grows from
he periphery and the peripheral region at any parti-
ular time becomes more central as the eye grows, so
hat the peripheral cone pattern must change into the
haracteristic central cone arrangement. The derivation
f the more central arrangement from that found at the
dge can be traced in tangential sections of the retina.
he basic cone pattern at the periphery consists of
arallel rows of double cones arranged with their long
iameters parallel to the edge. One consequence of the
arallel arrangement of double cones at the edge of the
etina is that in longitudinal sections they are always cut
t right angles to their long diameter and thus appear
ingle, which gives rise to the view that double cones
re absent at the edge of the retina (Verrier, 1928). A
arallel arrangement of double cones was observed by
lüller (1952) at the nasal edge of the retina in *Lebistes*,
nd Shafer (1900) describes a nearly parallel arrange-
ent of double cones at the edge of the retina of
licropterus, but these were arranged at right angles to
ne edge.

The parallel arrangement of double cones found at the
eriphery of trout retinae gives rise to the square pattern
f more central regions by a series of positional changes
rhich are shown diagrammatically in fig. 5, A–E. At the
xtreme edge, the double cones are arranged so that in
ach row parallel to the edge the orientation of the
vo halves of each double cone is the same, but is
pposite to that in the rows on each side (figs. 5, A and
, A). The single cones are arranged in rows between
ne double cones and are not always visible in the outer-
ost sections. Every alternate single cone is smaller
nan the others and these represent the additional
ngle cones of pattern II. If pattern I were formed
irectly from the parallel arrangement of double cones, the single cones
ould only be present at alternate intervals between the double cones.
have found this arrangement at the edge of a perch retina. The typical
entral trout cone pattern is derived from the parallel double cone arrange-
tent by movement of the double cones so that each row becomes zigzag
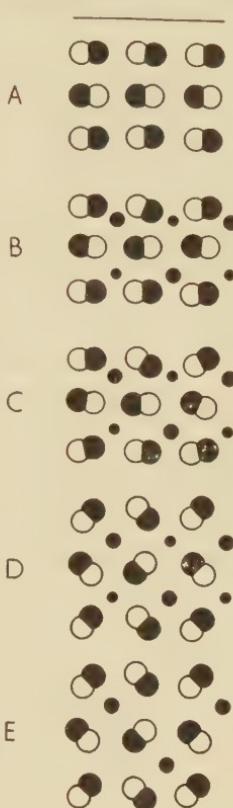
FIG. 5. Diagram of the changes in the cone pattern between the edge and centre of a trout retina. The horizontal line at the top of the figure represents the edge of the retina.

FIG. 6 (plate). A, tangential section cut near the edge of a trout retina showing the cone rrangement.
B, tangential section of a minnow retina showing the peripheral arrangement of cones.
C, part of a longitudinal section of a trout retina showing an element which may be inter-
ediate between rods and cones.

(fig. 5, C, D) till finally two adjacent double cones in the original rows are at right angles to each other (fig. 5, D; pattern II). The additional single cones are lost during later growth to give pattern I (fig. 5, E) which is characteristic of an adult trout retina.

The minnow retina is characterized by the numerous triple cones which it contains in addition to quadruple, double, and single cones. There is no regular cone pattern except at the edges, where only double and single cones are present and the double cones are arranged in parallel rows. It seems reasonable to assume that the minnow retina grows from the edge, like that of trout, and therefore the regular pattern of double and single cones will develop into the irregular arrangement of triple and quadruple cones characteristic of more central regions. A plan whereby this transformation may take place is shown in fig. 7. The arrangement of the double cones in rows parallel to the edge of the retina appears similar to that found at the edge of trout retinae, but the orientation of the two halves of the double cones is different. In each row parallel to the edge, the two halves of succeeding double cones are alternately arranged so that two similar halves (two light or two dark) of adjacent cones face each other (fig. 7, A). Adjacent rows have the opposite arrangement so that two dark halves face each other next to two light halves in adjoining rows. Considering the rows of double cones running at right angles to the edge, the orientation of the two halves alternates in succeeding double cones, as is also found in trout (compare figs. 5, A and 7, A). Rows of darkly stained single cones are present between the rows of double cones (figs. 6, B; 7, B). Alternate single cones are larger than the others, as in trout, and in some sections only these larger cones are visible.

The triple cones near the edge of the retina are usually still arranged in rows and often alternate with double cones. It appears from this arrangement of cones in minnow retinae that triple cones are formed by the fusion of a double cone with a single cone. If the large single cones were the first to fuse with the double cones this would give the alternate triple and double cone arrangement which is sometimes observed (fig. 7, C). As the other single cones grow larger they will also fuse with the remaining double cones so that at this stage the original pattern is lost, and the triple cones become irregularly arranged (fig. 7, D). A quadruple cone is probably formed by the fusion of another later developing single cone with a triple cone (fig. 7, E); some may also be formed by irregularities in the fusion with double cones so that some single cones fuse with the triple cones instead of with double cones.

The typical pattern in pike is formed of triangular units, but it is also derived from parallel rows of cones at the edge of the retina. The position of the single cones in relation to the double cones differs from that in trout and minnow, the most common arrangement being that shown in fig. 8, A. The change of pattern can be traced and is represented in fig. 8, A–D. The single cones are usually situated in the double-cone rows at regular intervals and the double cones lying between two single cones of adjacent rows are reorientated to lie at right angles to the double-cone rows (fig. 8, B). The positions of the other

ouble cones change, adjacent cones turning in opposite directions to form
e characteristic pattern (fig. 8, D). No difference in staining properties
etween the two halves of a double cone was observed in pike.
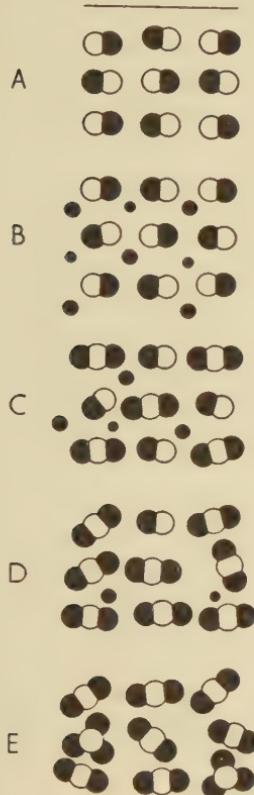
FIG. 7. Diagram of the changes
in the cone arrangement between
the periphery and centre of a
minnow retina.
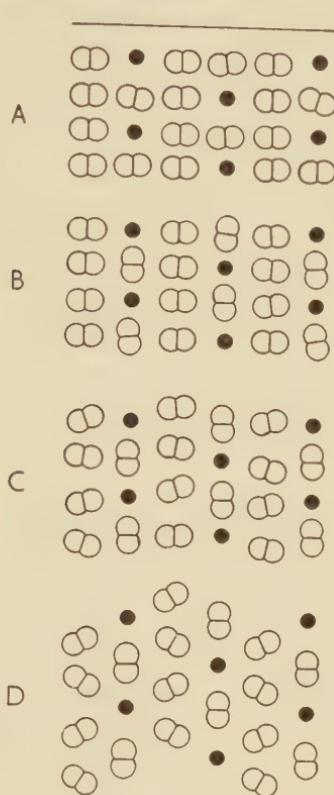
FIG. 8. Diagram of the changes in the
cone pattern in a pike retina.

I have always found the parallel arrangement of double cones at the edges
minnow retinae but not always in trout; this may be due to the fusion of
nes in minnow in addition to reorientation and the different times taken in
e two species to complete the changes. If the change of pattern in trout is
pid, the change-over zone will be narrow and more difficult to locate. It is
so possible that reorientation in trout may sometimes occur at the nuclear
age, before differentiation.

he transmutation of cones

The change of pattern found in trout retinae involves the loss of many
ngle cones; and the transmutation of these cones into rods seems the most
obable explanation of their disappearance, for there is no evidence of
generation, which appears to be the only alternative solution. There is

evidence of the transmutation of one type of visual cell into the other in several vertebrates, e.g. the rods of certain geckos resemble cones in structure but contain rhodopsin (Underwood, 1954). Various criteria have been used to distinguish between rods and cones, but, although the rods of one species may resemble the cones of another, within one species there is usually a clear distinction between rods and cones. Apart from differences in the size and shape of the inner segments, the rods and cones in a trout retina have clearly distinguishable nuclei which differ in shape, size, and staining properties. The cone nuclei are large and rather elongated and do not stain readily, whereas the rod nuclei are smaller, more spherical, and stain darkly. They also differ in their position in relation to the external limiting membrane, the rod nuclei all being situated wholly on the inner side of the membrane whereas the cone nuclei protrude through it so that generally two-thirds of each nucleus is on the pigment epithelium side of the membrane. I have found a few visual elements in trout retinae which may be intermediate stages in the transition from cone to rod (fig. 6, C). The nuclei of these elements stain like rod nuclei with haematoxylin or Mallory, and are similar in shape. The position of these nuclei in relation to the external limiting membrane is also rod-like, since they are generally situated entirely within the membrane and always with at least two-thirds of the nucleus on the inner side. A small, lightly stained myoid separates the nucleus from the cylindrical inner segment, which is narrower and shorter than that of a typical cone. A significant feature about these elements is that they are seen in longitudinal sections between two double cones cut through their long diameters, which is the same position as that of the additional single cones in younger retinae (compare figs. 6, C; 1, D). The retinae in which these elements were found belong to fish within the change-over size range, and it seems reasonable to assume that they are intermediate stages in the transmutation of the additional single cones to rods.

DISCUSSION

The teleost retina grows from a peripheral growth zone and it is in this region that the formation of the pattern is most likely to be seen. Müller (1952) devised a scheme for the development of the cone pattern in *Lebistes* (which is the same as that in young trout) based on the planes of mitoses in the growth zone. Müller examined the mitoses in surface sections and assumed that all the mitoses were concerned in the formation of cone nuclei, but, as mitoses are almost entirely confined to the outermost layer of nuclei in the growth zone, some of the products of division must develop into the other types of retinal cells which will eventually lie internal to the cones. I have examined the planes of mitoses in longitudinal sections of trout retinae and my results are in general agreement with those obtained by Glucksmann (1940) from tadpole retinae, in which there is no pattern. I found 76% of the mitoses parallel to the edge, 6% at right angles to it, and 18% intermediate at approximately 45° to the edge. Another difficulty with Müller's theory is that the pattern at the extreme edge of the retina may differ from that in more central regions.

which case the mitotic planes of the dividing cells in the growth zone can-
ot determine the final arrangement, for the peripheral region at any parti-
lar time becomes more central as the eye grows. It thus seems doubtful if
y interpretation of the mitotic axes can explain the formation of the charac-
ristic patterns. A parallel arrangement of double cones is probably the basic
ttern from which all others can be derived by positional changes and the
dition of single cones, but the origin of this basic cone arrangement remains
scure.

No functional significance has been attributed to cone patterns in teleost
tinae, but their frequent occurrence suggests that they may affect some aspect
visual perception. It is possible that cone patterns improve the perception
movement, since they are generally found in species which feed on fast-
oving objects. Bateson (1889) has shown that the majority of teleosts feed
sight, but some are more sensitive to movement than others. Movement
erception is generally of greater importance to predatory fish than high visual
uity. A cone pattern provides a uniform distribution of both types of cone
lls and this may be important if the single and double cones have different
nctions.

The relative distribution of single and double cones in teleost species sug-
ests that double cones are associated with vision in deep water, although
'under (1925), who examined 24 fresh-water species, found they were most
umerous in surface fish. In the Salmonidae, double cones are relatively more
umerous in deep-water forms than in species living in shallower water, e.g.
ar and gwyniad, which live in deeper water than trout and grayling, have
wer single cones than the latter two species, and the loss of the additional
ngle cones in trout and salmon may also be associated with their migration
to deeper water. Walls (1942) states that double cones alone occur in some
adus species, and I have found that there are almost exclusively double cones
the retinae of cod (*Gadus morhua*) and whiting (*G. merlangus*), both species
ving at considerable depths. The association of double cones with vision in
eep water may be due to greater sensitivity of the double cones so that they
e intermediate in sensitivity between single cones and rods, as Willmer
953) has suggested, or to differences in the spectral sensitivities of single and
ouble cones, since different wavelengths of light penetrate water to different
epths.

There are two theories on the origin of double cones in vertebrates. The most
idely held view (Bernard, 1900; Cameron, 1905; Detwiler and Laurens,
21; Eigenmann and Shafer, 1900; Müller, 1952; Saxen, 1954) asserts that
double cone is formed by the fusion of two adjacent cells. The alternative
eory, held by Dobrowolsky (1871), Howard (1908), and Franz (1913), main-
ins that a double cone is the result of incomplete division. It seems reason-
le to assume that all multiple cones are formed in the same way, thus triple
d quadruple cones will be formed in the same manner as double cones, and the
undance of triple and quadruple cones in minnow retinae supports the theory
at they are formed by fusion. As Saxen (1954) points out, two successive

incomplete divisions would be required to form a triple cone, therefore in minnow this would have to be a normal method of division. It is more probable that triple and quadruple cones are formed by the fusion of cone elements, and the changes in the cone arrangement observed between the peripheral and central regions of the minnow retina seem to support this view.

On the assumption that the peripheral arrangement of cones in minnow retinae develops into that of more central regions, there is evidence that double cones of equal size develop into unequal double cones. The double cones found at the edge have two halves of equal size with different staining properties, but during the formation of triple and quadruple cones one half of the original double cone must increase in size disproportionately to form the central cone. Most of the double cones present in the central regions of the retina also have one half larger than the other. Walls (1942) believes that as teleosts are a terminal group in evolution, non-teleost double cones cannot have evolved from teleost (equal) double cones, and the presence in teleosts of certain double elements with dissimilar halves may indicate incomplete equalization of the two halves. If, however, equal double cones had evolved by equalization from unequal double cones, it is unlikely that in the teleosts the latter would initially appear as equal double cones and only become unequal during later growth. The occurrence of the majority of unequal double cones among some of the most primitive families has been noted by Walls in discussing the evolution of teleost double cones, but it seems injudicious to consider the distribution of these elements in relation to the evolution of double cones when related genera may have different types of double cones. The distribution of equal and unequal double cones in teleosts cannot be correlated with the phylogeny of the fish: among the cyprinodonts *Fundulus* has unequal double cones (Butcher, 1938) but *Lebistes* has equal double cones (Müller, 1951), and similar differences are found in the Cyprinidae. The simplest theory of the evolution of double cones is that unequal double cones have evolved from equal double cones by the two halves becoming increasingly dissimilar, and the differential staining of some teleost double cones may represent the first stage in this evolution.

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The Contractility and Healing Behaviour of Pieces of *Leucosolenia complicata*

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With two plates (figs. 2 and 3)

SUMMARY

The behaviour of isolated pieces of *Leucosolenia complicata* confirms that this sponge contractile. The pieces first curl up owing to a contraction of the internal epithelium, hich can exert a tension in one direction (transverse) more than the other at right ngles. The contraction is slow because it entails a redistribution of the supporting esogloea. It is abolished by 5 minutes' treatment with distilled water.

Healing next involves the formation of 'healing membranes'. Each membrane con-sts of two epithelia with a thin layer of mesogloea in between. The membranes arise ither as an outfolding of the internal epithelium or from the cut edges of the wall, and ney spread between the edges so that the tubular form of the olynthus is regained. heir spread is due to the maintenance of tension in the membrane, coupled with the hrinkage of the remainder of the piece.

Pieces from which the internal epithelium has been brushed away shrink rapidly nd become saddle-shaped, indicating that the dermal epithelium also is contractile.

No important differences are noticeable when the healing behaviour takes place in qual parts of sea-water and isotonic magnesium chloride, which suggests that the ehaviour is not under the control of a nervous system.

A discussion is given on the elements responsible for the contractility of the internal pithelium. It is probable that the porocytes are connected beneath the bases of the ollar-cells, and are the contractile cells concerned.

CONTENTS

INTRODUCTION

DURING an investigation of the mechanism of orientation of calcareous sponge spicules a number of experiments were attempted in which part f the wall of an oscular tube of *Leucosolenia variabilis* was removed and re-laced in the reverse orientation. Some of the attempts were successful and ndicated that the newly forming spicules were not obliged to copy the orienta-ion of their older neighbours, but in other cases the piece fell away, whereupon oth the piece and the mutilated tube underwent a process of healing by

which the functional tubular form was regained. Since the process invariably involved a curling of the wall, a study was made of the healing behaviour to see whether it would throw light on the contractile properties of the sponge.

The contractility of calcareous sponges, in particular *Clathrina coriacea*, has been known for many years. The pores and oscula can be closed and the whole sponge can undergo a general process of contraction. Bidder (1898) believed that this was simply a recovery from the stretched condition after the cessation of activity of the collar-cells; he thought that normally the internal hydrostatic pressure gave rise to a state of tension in both internal and external surfaces of the sponge. However, Minchin (1900) states that 'the flat epithelium covering the exterior is responsible for the general contraction of the whole body', and that 'the closure of the osculum is effected more especially by . . . cells . . . which line the oscular rim'. The latter cells often give rise to 'a special contractile apparatus, such as a ring-like sphincter, or a contractile sieve-membrane', and, as Minchin points out, this suggests that any contraction in the vicinity is more than an elastic recoil. The sieve-membrane of *C. coriacea* is certainly contractile, since a tube can become constricted in this region, without either the oscular edge itself, or the rest of the tube, becoming much contracted. Such a sieve-membrane is not to be seen in *Leucosolenia complicata*, but a diaphragm may be present as a trilaminate shelf around the inner surface of the oscular rim (Jones, 1954*b*). Furthermore, the osculum of this species has been observed to contract in bright light, the area of contraction passing as a wave around the oscular rim. Minchin (1908), however, states that 'the species of *Leucosolenia* are very slightly, if at all, contractile'.

The experiments described in this paper also have some relevance to the problem of whether or not there is a nervous system in *Leucosolenia*. Recently Tuzet, Loubatières, and Pavans de Ceccatty (1952) have affirmed their belief in the existence of a nervous system in sponges, including *L. botryoides* (Pavans de Ceccatty, 1955), as a result of their studies of stained sections. Such a nervous system, if present, should be capable of narcotization, and for this reason an investigation has been made of the healing behaviour of pieces of *L. complicata* in a solution containing equal parts of sea-water and isotonic magnesium chloride. This solution narcotizes the neuromuscular system of *Metridium senile* after about 1 hour (Batham and Pantin, 1951).

MATERIAL AND METHODS

The healing behaviour of three species of *Leucosolenia* has been studied, namely *L. complicata*, *L. botryoides*, and *L. variabilis* (Minchin, 1904), but most of the work has been done with *L. complicata*. Specimens were kept under running sea-water for several days or weeks and then transferred to the laboratory, where some of the oscular tubes were removed. From these, pieces were derived by bisecting the tubes longitudinally with fine scissors. The pieces were then placed in Petri dishes containing 40 ml of sea-water at room temperature (usually 15–20° C), and kept under low-power observation.

Measurements were made by means of a squared eyepiece-micrometer. The longitudinal bisection of the tube afforded a control half for experimental purposes when necessary.

Some pieces were fixed in osmium tetroxide dissolved in sea-water at different stages of the healing processes, and stained with picrocarmine (Minchin, 1898).

Other methods are mentioned below.

RESULTS

The behaviour of isolated pieces of the wall

Pieces obtained by the longitudinal bisection of the oscular tubes of *L. com-plicata* undergo in sea-water a process of repair and reorganization which can be divided into three main phases. The first phase involves the curling up of
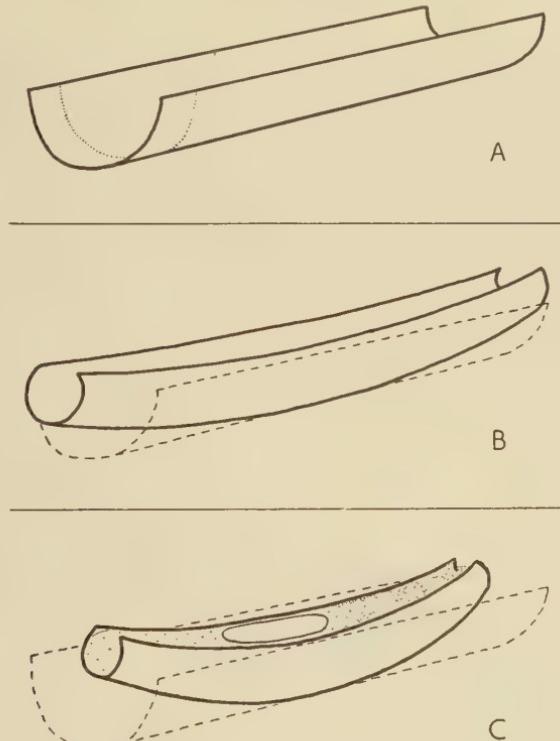
FIG. 1. Diagrammatic representation of the curling and healing behaviour shown by a piece of the wall of *L. complicata*. A, piece immediately after cutting. B, at the end of the first phase (curling). C, during the second phase (healing membranes). Description in the text.

the piece in the longitudinal and transverse planes, so that the choanoderm comes to line the cavity enclosed by the resulting canoe-shaped structure (fig. 1, B). The second phase begins with the formation at each end of the

piece of a 'healing membrane', consisting of two cellular layers with a thin
layer of mesogloea in between. It exhibits contractile properties, for it draws
the edges of the original wall closer together and spreads across the gap,
sealing off the cavity until a complete tube has been formed (fig. 1 C). The
final phase then follows with the growth of the tube into the normal form of
the species.

The first phase of curling invariably results in the partial enclosure of the
choanoderm and the original spongocoel, and takes place regardless of
whether the piece is left undisturbed with the choanoderm facing upwards or
downwards. It also occurs with pieces derived from the base of a tube ('basal
pieces'), without a portion of the diaphragm, or inturned porocyte epithelium
of the oscular rim. This suggests that the choanoderm is contractile, the
curling up being analogous to the curling of a photographic print when the
gelatine is shrunk by drying.

TABLE I

Measurements in micrometer-scale divisions of two pieces of the wall of L. com-
plicata *which were left resting on their dermal surfaces in sea-water. The pieces
were derived from the same oscular tube,* A *from the basal end and* B *from the
oscular end. The widths are the distances between the cut edges at the three levels.*
1 *micrometer division equals* 160 μ

	Piece A		Piece B	
	at		at	
	0 h	3½ h	0 h	3½ h
Width at:				
oscular end . .	6·2	4·8	3·6	3·3
middle . . .	6·5	6·3	5·9	6·1
basal end . . .	6·9	5·7	6·7	6·0
Length. . . .	9·6	9·3	9·5	9·8

The process of curling has been followed quantitatively by measuring the
length and the distance from edge to edge at each end and in the middle of
the piece. As can be seen from piece *A* (table 1), the length may decrease as the
piece curls upwards, but this decrease is at first probably only an apparent
one, the ends being drawn closer together with the increase in curvature. In
fact in several cases (e.g. piece *B*, table 1) an increase in the apparent length
was detected, and also in the true length, measured directly on pieces lying

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FIG. 2. Photomicrographs of fixed pieces of *L. complicata*, stained with picrocarmine. A, part
of the edge immediately after excision. The collar-cells and broken ends of the spicule-rays are
close to the edge. B, the lateral edge of a curled piece fixed 23½ h after excision. The collar-cells
and broken ends of the spicule-rays are now separated from the edge by a membrane which is
propped out by unbroken rays. Longitudinally stretched dermal cells may be seen at the points
indicated. C, the healing membrane at the oscular end of a piece fixed 24 h after excision. Note
the absence of collar-cells on this membrane. The line in each case represents 100 μ.
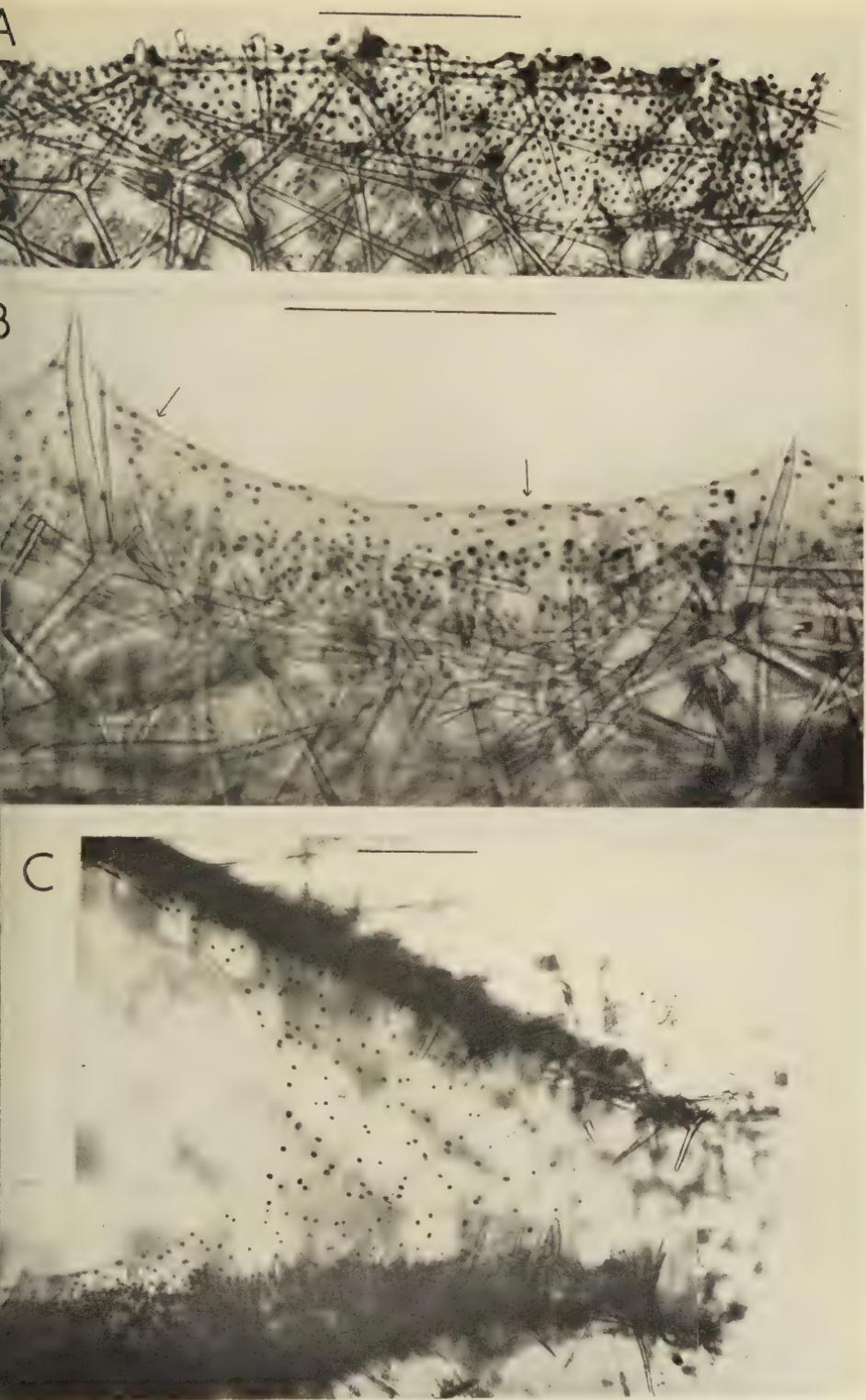
Fig. 2
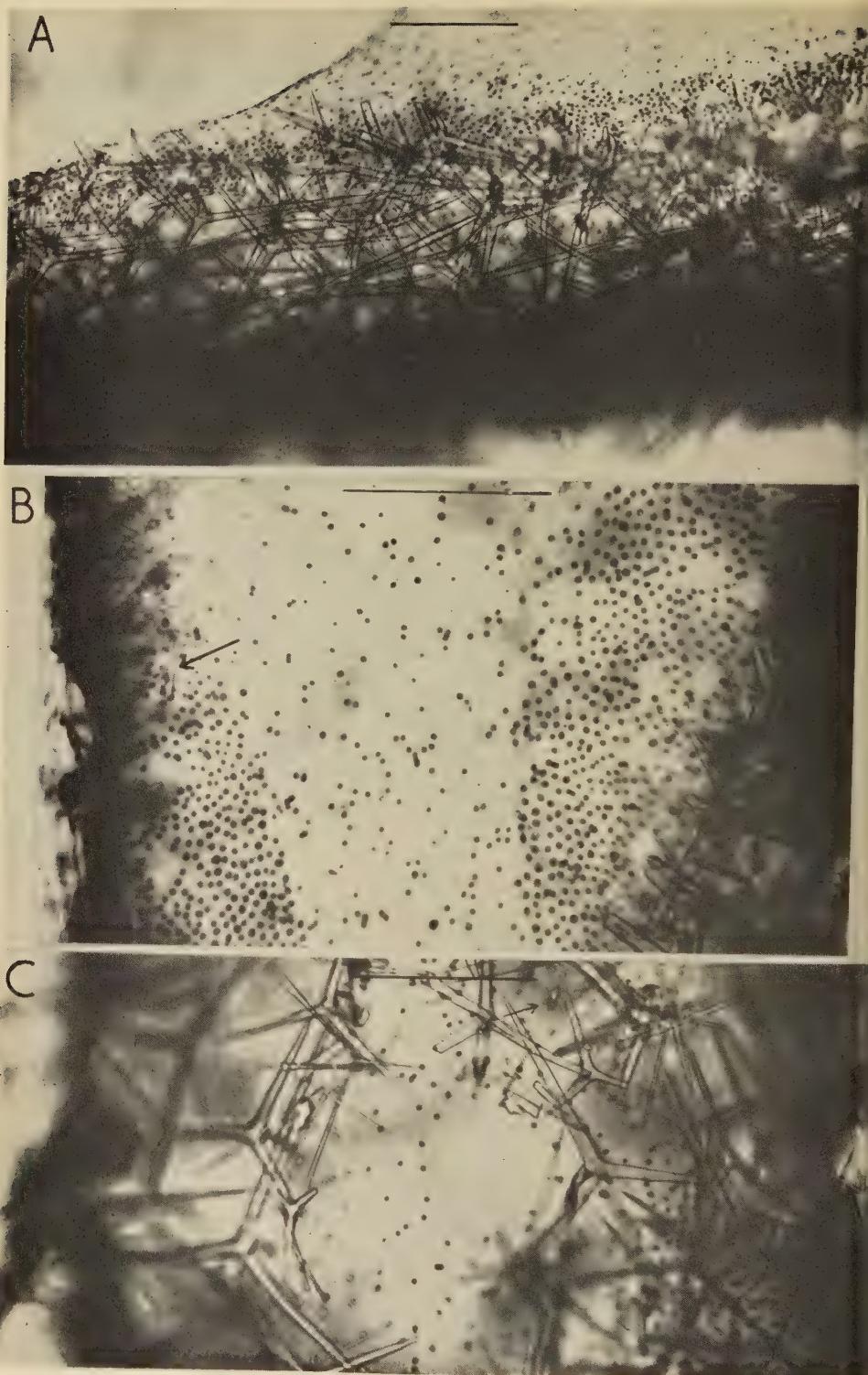
W. C. JONES

FIG. 3

W. C. JONES

n their sides, which indicates that the process of curling has been accompanied by a longitudinal extension of the supporting mesogloea.

Sometimes the edges may separate in the middle of the piece while at the ends they are moving inwards (piece *B*, table 1). This partial flattening in the middle is probably a mechanical effect necessitated by the longitudinal curling in such cases. There may also be a slight separation of the edges at the oscular end of 'oscular pieces' (having a portion of the oscular rim), or the process of incurling at this end may be delayed. This was observed in 4 out of 7 pieces measured, and in the remaining 3 the oscular end curled inwards relatively less than the basal end. With 'basal pieces', on the other hand, the tendency for the oscular end to uncurl was much less evident, only 1 piece out of 6 measured showing this effect. Any tendency for the pieces to uncurl immediately after being isolated, therefore, concerns the part derived from the original oscular rim and is probably to be correlated with the occasional arching of this part in the longitudinal plane towards the dermal side. Both tendencies indicate the presence of tension in the dermal epithelium. Their effects are only temporary, and they confirm that the collar-cells or functional horocytes are responsible for the normal curling behaviour since these cells are absent from the oscular rim.

During the first phase of the healing behaviour the cut edges of the piece become reorganized to form a clear zone which arches between the projecting spicule rays (fig. 2, B). The act of cutting leaves a fairly straight edge, with the collar-cells sometimes reaching close to it (fig. 2, A), but more often pushed back a little, or scraped away from it, by the blade of the scissors used. Here and there along the edge the collar-cells may be concentrated together in small areas by the action of the scissors, but there is no evidence for an immediate retraction of the choanoderm when the wall is cut. The collar-cells must either be firmly attached to their substratum, or else they are not under tension.

As curling proceeds some of the spicules near the edge become jammed against the interlocking rays of adjacent spicules and their rays then project more and more at the edge as the neighbouring spicules are drawn inwards by the process of contraction (fig. 2, B). The numerous quadriradiates are, of course, anchored to the choanoderm by their gastral rays, and it seems that the choanoderm contracts mainly in the transverse direction, drawing these spicules together where possible. This motion is opposed by the mesogloea to some extent, so that the wall curves more in the transverse plane, but some lateral squeezing of the mesogloea takes place and this necessitates the lengthening of the piece, provided the volume and thickness of the mesogloea

FIG. 3. Photomicrographs of fixed pieces of *L. complicata*, stained with picrocarmine. A, basal end after 23½ h. The collar-cells and pores are being dragged from the original edge of the piece. B, surface view of a healing membrane near the basal end of a piece 24 h after excision. Collar-cells, pores, and a spicule-fragment (arrowed) can be seen. C, stage just before the completion of healing. Only a small hole remains. Note that no nuclei are present at the rim of the hole. A newly forming triradiate (arrowed) and several slender monaxons are visible. In each case the line represents 100 μ.

do not appreciably change. The tendency for the mesogloea to extend against the longitudinal tension in the choanoderm assists in the curling of the piece in the longitudinal plane, and thus the whole process of curling is explicable in terms of the predominantly transverse contraction of the inner epithelium. This contraction indicates that the epithelium responsible is anisotropic, for it can shorten in one direction whilst extending in the direction at right angles. The contraction, however, need not strictly be transverse, and quite possibly the tension is developed on a spiral course, the spirals conforming to the spiral organization of the oscular tube (Jones, 1955).

As some spicule rays project more and more laterally they prop out the dermal epithelium (fig. 2, B). In this figure the dermal cells near the edge can be seen to be stretched in the longitudinal direction, as would be expected from the above explanation concerning the elongation of the piece. The projecting rays disturb the orientation of the cells somewhat and add to the amount by which the cells in the vicinity are stretched.

The clear zone comprises two layers of cells with a thin layer of mesogloea in between. Each epithelium consists of flattened cells with polygonal outlines that can be faintly seen on living specimens. The cells of the inner surface may be derived from the outermost choanocytes by a process of flattening and spreading, such as has been described by Duboscq and Tuzet (1939), but there is little evidence for this; almost everywhere along the edge the collar-cells are sharply demarcated from the flattened epithelium and very few cells that could be regarded as intermediate in form can be seen. Thus it is more probable that, as the spicule rays are left projecting by the contraction of the choanoderm, part of the dermal epithelium becomes pulled over the edge in between these rays. Alternatively, the inner layer of the clear zone is produced from amoebocytes in the mesogloea.

The first phase of healing may be regarded as ending with the appearance of healing membranes at the oscular and basal ends of the piece. A membrane is usually seen first at the oscular rim and this seems to be identical with the diaphragm sometimes present within the rim of complete, but possibly not fully expanded, oscular tubes. The membrane may arise at the extreme edge of the piece, or from the surface of the porocyte epithelium, or from the junction between this epithelium and the choanoderm. In the pieces observed it became conspicuous at times ranging from $7\frac{1}{2}$ to 19 h (fig. 2, C). A similar membrane is formed at the basal end, although usually a little later (fig. 3, A). Both membranes are capable of drawing the edges of the piece closer together. They advance towards each other, merging on each side with the lateral membranes that arch between the projecting spicule rays, until only a circular or oval hole is left in the mid-line (fig. 2, C). This hole usually lies nearer the oscular end owing to the more rapid advance of the basal membrane. It may become completely obliterated (sometimes within 24 h after excision) after serving as an osculum for a time. Before this currents can be detected leaving through the hole, confirming that the flagella remain active. The pores on the original wall stay open throughout.

The basal healing membrane arises close to the basal end of the piece as a shelf projecting inwards from the inner surface. As with the diaphragm, its formation appears to be the result of tension developing in a transverse band or line of cells across the inner surface, and its spread is presumably due to the continued maintenance of tension at the free edge. The cells certainly are stretched along the edge, as seen in optical section, except perhaps when the gap is practically sealed (fig. 3, c); but what happens to them as the gap closes and the edge diminishes in extent is still a problem. According to Minchin (1900) the dermal cells become mushroom-shaped when they contract, the cell-bodies moving into the mesogloea. Maas (1910) agrees with this observation. Cells of this shape, however, have not been seen at the edges of healing membranes on fixed pieces, but possibly their withdrawal from the edge is a relatively rapid affair, so that few examples would be present at the moment of fixation. On the other hand, since the area of the original piece continues to shrink in both length and width as the membranes spread across the gap, there may be no need for an alteration in the total number of cells in the epithelia. This would imply that the cells are capable of losing contact with the adjacent cells and making contact with their new neighbours without leaving the epithelium, since the pattern of distribution of the cells must change as the membrane obliterates the gap.

While it would appear that tension is developed at the free edge, the membrane as a whole is tending to contract and draw the edges of the piece closer together. This process results in the collar-cells being dragged beyond the limits of the original wall, and spicule fragments may accompany them (figs. 2, c; 3, b). The spicules themselves, however, remain and become crowded together as the piece shrinks. No doubt the sharpness of the angle between the membrane and the original wall precludes the spicules from being drawn across with the collar-cells. The interlocking of the spicule-rays gives support to the original wall and prevents the inturning of the edges, so that as the healing membrane spreads across the gap, the inner and outer epithelia of the original wall are drawn beyond the limits of the shrinking area of spicules. Presumably the cells of the epithelia part and rejoin to allow movement past the gastral and other projecting rays.

The result of the healing process is usually a small, thin tube, which is often crooked, particularly if the original tube from which the piece was obtained was bent, or not bisected symmetrically. Sometimes one end of the piece becomes bent over the other end, producing a spiral, while at other times the two ends meet to form a ring. The healing membranes then spread across the gaps bounded by the lateral edges, forming spheroidal objects or coiled tubes.

Newly forming spicules become visible in the healing membrane after about 20 or more hours. Slender monaxons appear first, lying in between the two surface epithelia and often in contact with the inner one. They tend to be oriented at right angles to the free edge of the membrane, but later (after 5 days), when the piece has healed completely, they are pushed through the outer dermal layer and project from the surface. Young triradiates, which may

also continue to grow on the original wall, make their appearance within the membrane after about 2 or more days, developing on the inner epithelium. Their orientation quite often appears to be directed towards the free edge of the membrane, but the arrangement is more often than not confused, which is to be expected from the time taken for these spicules to develop (over 1 day), for the direction of the free edge in relation to the growing spicule may be constantly changing as the membrane spreads across the gap. Young spicules may also be dragged on to the membrane from the original wall as the piece shrinks, and their orientation will be disturbed if, as seems likely (Jones, 1952), it is caused by the operation of mechanical factors. Abnormal conditions of development are demonstrated by the large number of aberrant spicules that are produced under these conditions. Some arise in formative complexes with an unusual arrangement or number of calcoblasts. For example, tiny triradiates have been seen in groups of 4 and 5 cells, instead of the normal 6, while sometimes no calcoblasts may be present around a small primordium, the cells presumably having departed or been torn apart. In some sextets the calcoblasts are arranged in the form of a rosette, whereas in others the outer 3 cells are displaced with respect to the inner 3 as in some of Minchin's drawings (see Jones, 1954a). Since abnormal spicules develop in the membranes, the latter are not so suitable for studying the normal development of the sextets, although some growing spicules do show a perfect bilateral symmetry, with the 3 thickener cells in the angles between the rays and the 3 founder cells at the ray-tips (Minchin, 1908).

Histological examination of fixed membranes confirms that the membrane consists of two epithelia with a thin layer of mesogloea in between. In *L. botryoides* the nuclei of the outer dermal layer may be larger than those of the inner. No conclusive signs of cell-division have been observed in the dermal epithelia. The collar-cells form a continuous sheet apart from the porocytes, which may be open or in the process of opening. Collars and flagella are clearly visible on the preserved choanocytes. There is little evidence of a transformation of collar-cells into flattened cells, although at the border of the choanoderm one may see choanocytes with their granular cytoplasm more spread out. Generally, however, the area of the collar-cells is sharply demarcated from the rest of the membrane.

The mesogloea contains amoebocytes and calcoblasts in contact with the inner epithelium. Spherical cells with a peripheral zone of large granules (the 'excretory cells' of Minchin, 1908) are quite common on or in the membrane.

Examination of the healing membrane under the polarizing microscope reveals no birefringence when the membrane is seen in surface view, but in optical section the membrane glows and darkens four times in a complete 360° rotation. This is best seen when the membrane has completely healed over the gap and the piece is resting on one side. No birefringence is detectable on membranes fixed and mounted in balsam. The birefringence seen in optical section when the medium is sea-water probably arises from the layering of the material in the cell-walls of the flattened and closely set epithelia.

The third phase of the regenerative behaviour, involving the opening of an osculum and the growth of the olynthus, has not been followed far, but there can be no doubt that, provided the piece is supplied with nourishment, the normal specific form will be attained.

It should be noted that the first two phases of the healing process are essentially distinct. In the first, the piece curls owing to the predominantly transverse contraction of the internal epithelium, with little or no reduction in the mesogloeal volume. In the second, both internal and external epithelia spread across the gap mainly as a result of the contraction of the free edge, while the original wall, as represented by the area of fully-grown spicules, shrinks considerably. The importance of the spicules to both processes is, however, obvious. The gastral rays assist in the curling because they anchor the quadriradiates to the internal epithelium; and the other rays, interlocked together, provide the necessary rigidity to enable the healing membranes to spread across the gap without the gastric cavity becoming obliterated.

Mutilated tubes that are not removed from the specimen show the same kind of healing behaviour as the isolated pieces. For example, when distal longitudinal slits are made at diametrically opposite positions in the wall of an oscular tube and half of the cylinder is then excised, the remaining half usually arches at first towards the dermal side, either as a result of longitudinal tension in the dermal layer, or owing to the pressure of the outflowing current of water; but then invariably a phase of inward curling takes place. The part bends right across the outflowing current and the free corners close together until they have met and fused, making the gap in the tube triangular. Healing membranes then spread across the gap from the three angles and the tube constricts and remoulds itself distally, until the cut edges have been brought as close together as possible. Elsewhere spicules form in the healing membrane. Thereafter, with the normal growth of the tube, the distal kink becomes somewhat smoothed out as the tube lengthens and increases in girth.

The same occurs when the oscular rim has been excised before removal of the rectangular piece: the presence of the porocyte epithelium is thus not essential for the inward curling. Also with other types of mutilation the behaviour is essentially the same; there is a phase of inward curling, followed by the spread of the healing membrane and the shrinkage of the original wall.

Pieces of the wall that have been immersed for 5 min in distilled water (or derived from a tube treated in this way) and then replaced in sea-water, sag and eventually flatten against the bottom of the dish. Subsequently they shrink in both transverse and longitudinal dimensions and continue to do so for several days, becoming more flexible at the same time. In one specimen the mesogloea became noticeably thicker. There was some variation in the total shrinkage (10–20% in 5 days) and also in the time at which shrinkage first became apparent (1–12 h), but the rate of shrinkage eventually decreases with time, probably as a result of the crowding together of the spicules. The cause of the shrinkage has not been ascertained, but it appears to involve the softening of the mesogloeal substance, possibly by bacterial activity.

Pieces that are left in distilled water also sag, though occasionally the oscular end may incurl as the basal edges flatten. However, this effect is not sustained. A very slight shrinkage occurs and the spicules corrode and have disappeared after about 3 days.

Thus the changes in shape which the healthy pieces undergo in sea-water depend on the presence of the epithelia. The cells are destroyed by the 5 minutes' treatment with distilled water, whereas the colloidal properties of the mesogloea are not appreciably modified (Jones, 1956).

The behaviour of pieces after removal of their choanoderm

The facts given above suggest that the curling is caused by a contraction of the inner layer working against the relatively firm mesogloea. In order
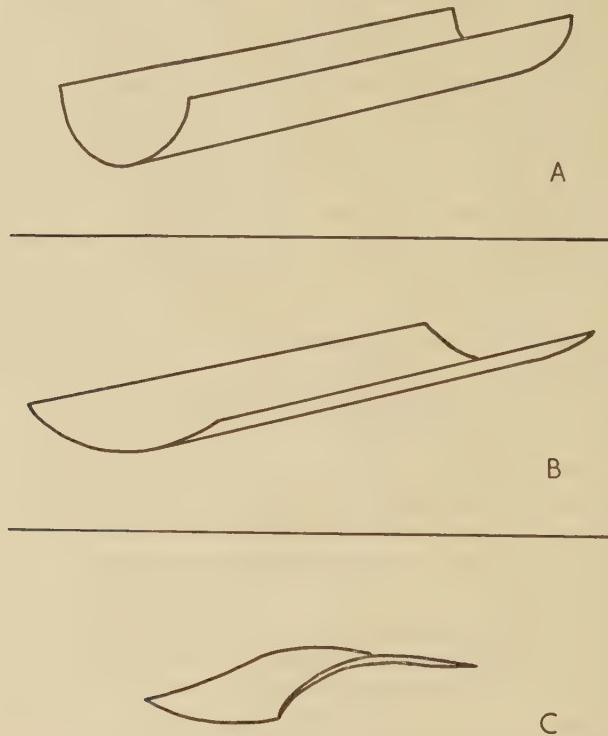
FIG. 4. Diagrammatic representation of the changes undergone by a piece of *L. complicata* after the removal of the internal epithelium by repeated brushing. A, the piece immediately after cutting. B, partial recovery of curvature after brushing. C, shrunken and saddle-shaped condition produced after a few hours

to test this, longitudinal halves of oscular tubes were held at one end by means of a needle and brushed repeatedly on their inner surface. The end damaged by the needle was then cut away and the behaviour of the brushed pieces was compared with that of the control halves. The latter underwent

he normal process of curling and healing, even after their dermal surface
ad been brushed for the same number of times as had the inner surface of the
ther halves. The pieces without their choanoderm, however, after partially
ecovering their initial curvature (for they are flattened by the brushing),
apidly shrank and became saddle-shaped (fig. 4, c). The resulting appearance
idicates that the pieces had curved towards the dermal side in the longitudinal
lane, while decreasing their transverse curvature at each end. Table 2 shows
ie typical changes in size of a piece brushed on the choanodermal side. The
nanges in width are due to shrinkage and not to curling inwards in this case,
hile the changes in apparent length are due partly to the arching towards the
ermal side and partly to shrinkage. The rate of shrinkage decreases as the
picules become closely packed together. At $43\frac{1}{2}$ h the piece had lost its
ongitudinal curvature, which accounts for the apparent increase in length.

TABLE 2

*iece brushed well on its choanodermal side and left in sea-water. Measurements
as in table 1. Explanation in the text*

| | | | Piece C | | | | |
| | | | | at | | | |
	o h	5–6 h	$6\frac{1}{2}$ h	19 h	$43\frac{1}{2}$ h	68 h	$92\frac{1}{2}$ h
Vidth at:							
oscular end . .	5·5	4·5	3·4	3·2	3·3	3·3	3·3
middle . . .	5·0	4·4	3·8	3·3	3·5	3·7	3·7
basal end . .	4·7	4·0	3·2	2·8	3·1	3·4	3·4
ength . . .	14·9	13·0	12·2	10·8	12·7	12·3	12·7

As the brushed pieces shrink, a few large circular gaps form in the meso-
loea between the spicule-rays. They may be derived from cavities originally
ccupied by porocytes, or possibly from small holes made by the bristles of
ie brush. These have been seen on pieces fixed immediately after the brush-
ig. The enlargement of these cavities (which may reach 30 μ or more in
iameter) and the very rapid shrinkage suggest that the mesogloeal substance
as been softened and possibly reduced in volume.
The saddle-shape appears to be the result of a contraction of the dermal
urface, possibly at the edges only, but in any case opposed by the inter-
ocking rays of the spicules. The paired rays of the triradiates do not lie in the
ame plane (Jones, 1954b), but are inclined so as to embrace partially the
scular tube. The overlapping of the rays of a number of adjacent spicules
ould hence hinder any process tending to curve the wall transversely towards
ie dermal side, especially after the wall has shrunk and the spicules have
ecome crowded together. Thus while a limited amount of longitudinal
urling towards the dermal side is possible from the original straight condition,
ie contraction of the dermal layer merely results in a partial uncurling
ansversely at the ends of the piece. In the middle the piece remains markedly

curled in the transverse plane, thereby accommodating the longitudinal curvature towards the dermal side of the lateral edges of the piece.

It is necessary to brush the pieces really well (over 50 strokes with a camel-haired brush) for the above to occur, for if the brushing is not sufficient to remove most of the collar-cells and porocytes, the pieces still tend to curl in both transverse and longitudinal planes to the original choanodermal side, although the curling is not so striking as with the controls. Such pieces usually have small islands of collar-cells remaining, and open pores and tiny spicules may be seen in the regions from which the collar-cells have been removed. The collar-cells tend to spread themselves out somewhat, and the internal epithelium becomes reorganized and causes the curling behaviour. In such pieces healing membranes develop at the ends and laterally, but they appear usually some distance from the original edges of the wall and at a later time than in the unbrushed pieces. Such membranes have even been obtained with well-brushed pieces, and by their contraction and spread they can induce a reversal of the longitudinal curvature and form a complete tube, roughly elliptical in cross-section, along the centre of the piece.

Thus, provided the brushing has been so severe that the porocytes and other cells associated with the collar-cells are largely removed, the normal process of curling does not take place. One must not therefore overlook the possibility that the porocytes are responsible for the contractility of the inner surface. Furthermore, the production of the saddle-shape indicates that the outer dermal epithelium also is contractile.

The behaviour of pieces in sea-water mixed with $MgCl_2$ solution

When pieces of *L. complicata* are left in a mixture containing equal parts of sea-water and isotonic magnesium chloride ($7\frac{1}{2}\%$), no significant difference in behaviour can be detected from that occurring when ordinary sea-water is used. In both media the pieces always curl up with the choanoderm on the inside, often elongating as they do so. Some unimportant differences were noticed, however. Thus in the $MgCl_2$ sea-water the formation of a healing membrane was sometimes delayed, although never prevented (4 pieces out of 7). Also the spicules showed signs of corrosion after 24 h and new spicules did not grow in the healing membrane. The flagella also seemed to beat more actively (judging by eye), and 'blisters' developed on 4 pieces in the mixture compared with only 1 on the controls in sea-water. The development of blisters is interesting, since Maas (1910) believed that they were caused by starvation, which would be more likely in the artificial medium. However, the contraction of the internal epithelium and the formation and spread of the healing membranes are certainly unimpaired in the $MgCl_2$ sea-water.

Now the presence of a nervous system in *L. botryoides* and other sponges has been claimed by Pavans de Ceccatty (1955) as a result of his study of mesogloeal cells that resemble the neurones of higher animals. These cells are dissimilar, however, in being interconnected asynaptically, but they are described as making synaptic connexions with other types of cell. One would

pect that such cells, if they are nervous in function, would play a part in the
behaviour of the sponge, and also that their action would be modified in the
presence of $MgCl_2$ sea-water, for the magnesium ion is believed to act on cell-
surfaces (Danielli, 1950), reducing their excitability. For example, $MgCl_2$
sea-water completely inhibits the neuromuscular system of the coelenterates,
and yet the curling and healing behaviour of *Leucosolenia* takes place in this
medium. There is no reason to suppose that the ions of magnesium chloride
do not penetrate into the mesogloea, particularly as tubes left in isotonic
magnesium chloride become softened after only 11 h (Jones, 1956). One can
only assume, therefore, that either the 'neuromuscular system' of *Leuco-
solenia* is unaffected by the magnesium chloride, or the 'nervous system' does
not play a significant part in the behaviour of the sponge; but neither alterna-
tive enables one to accept with confidence the claim that a nervous system is
present in *Leucosolenia*.

Discussion

The process of curling of the isolated pieces indicates that the internal
surface is contractile, and since this surface directly opposes the excess
internal hydrostatic pressure one can assume that it is normally in a state
of tension. Whether this tension is the result of a tonic contraction of the
epithelium, as Dr. C. F. A. Pantin has suggested (private communication), or
whether it is due to the elastic stretching of the material, as Bidder (1898)
believed, cannot be decided on the evidence presented. However, the existence
of tension of one form or another is shown by two observations. Bidder (1898)
noticed that flagellated chambers often turned themselves inside out when
eased, and concluded from this that there was elastic matter just beneath
the collar-cells, while Huxley (1912) likewise noticed the tendency for blocks
of collar-cells to change the direction of curvature, and attributed this to
tension existing in the interstitial substance at the base of the cells, or to
tension in the epithelium as a whole. He did not notice any other types of cell
associated with these blocks. The blocks of collar-cells eventually rounded
themselves off into spheres, and Huxley explained this by the tendency of the
cells at the periphery to draw themselves into closer contact with their neigh-
bours. This, if correct, would indicate that the collar-cells are capable of
developing tension, but other evidence favours the porocytes as the elements
responsible for the contractility of the internal surface.

Dendy (1890) noticed that in *Grantia labyrinthica* the inner layer of some
of the radial tubes had shrunk, the collar-cells forming a multilayered mass
in the centre of the tube and the mesodermal cells being pulled out into
strands forming a radially-disposed network serving to suspend the central
mass. This observation has been confirmed and extended by Duboscq and
Tuzet (1939). They point out that the collar-cells of *Sycon raphanus* rest on
a very thin membrane of collencytes and that when contraction occurs the
collar-cells become vespiform in shape. From their bases rooting processes
join up with the processes of the underlying mesodermal collencytes, some of

which are radially disposed. The latter spread out distally at the level where the vespiform bodies of the collar-cells appear to make contact together, and at this level eosinophil granules which were present in the cytoplasm of these collencytes could be seen between the collar-cells. This implies that it is the collencytes forming the membrane on which the choanocytes rest that are the contractile elements, and that their contraction results in the membrane moving inwards, with the consequent stretching of the radially-disposed collencytes and the pulling of the anchored bases of the choanocytes through this membrane. Dendy's observation of the piling up of the collar-cells to form several layers is consistent with the view that the collar-cells are not the contractile elements.

Now Minchin (1900) has described the inward migration of the porocytes when *Clathrina coriacea* contracts completely, while Maas (1910) gives a similar account for *Leucosolenia lieberkühnii*. The collar-cells first become narrow and elongated, and then pile up on one another, while the porocytes pass between them and come to form 'an epithelium lining the now greatly reduced gastral cavity' (Minchin, 1900, p. 30). Such a process could be explained if one assumed first that the collar-cells are anchored to the parts beneath the choanoderm, and secondly that the porocytes are interconnected by cell-processes which pass between the bases of the collar-cells. Contraction of the porocytes together would then result in the choanocytes piling up, slipping through the gaps, and eventually coming to lie outside the porocyte mass. The porocytes of *Clathrina* and *Leucosolenia* would then be analogous to the collencytes which form the membrane beneath the collar-cells of *Sycon*, and would constitute the tense, contractile system of the internal layer of the normal oscular tube.

The assumption that the porocytes of *Leucosolenia* are responsible for the contractility of the inner surface is an attractive one. The porocytes are obviously contractile, since they can occlude their pores, as Minchin (1900) has pointed out. Furthermore, they are derived from the inner edge of the porocyte epithelium lining the oscular rim (Minchin, 1898), from which also the obviously contractile diaphragm is produced. This has been fully confirmed by means of photographic records of the growth of oscular tubes of *L. variabilis* (Jones, 1952). These demonstrate the formation of pores at the junction between the porocyte epithelium and the choanoderm as the limit of the collar-cells advances, keeping pace with the longitudinal growth of the tube. The choanocytes apparently move around the newly-opening pores (Minchin, 1898) and this suggests that they are only loosely contiguous and that they are freely mobile either on the mesogloeal surface, or on the extensions which probably persist between the porocytes after the pores have opened. The movement of the collar-cells does not suggest an epithelium under tension; more likely the collar-cells mutually compress each other, since the division of the collar-cells is presumably causing the limit of the choanoderm to advance.

Histological evidence for interconnexions between the porocytes is rather

anty. Prenant (1925) observed that in the fully-expanded specimens of *lathrina coriacea* the porocytes were insinuated between the choanocytes tuated around their border, which suggests that there might be processes om porocyte to porocyte between the bases of the collar-cells. Minchin and eid (1908) observed a honeycomb network after removing the collar-cells d staining a piece of wall with picronigrosine; they believed that this was e interstitial substance between the bases of the choanocytes, but it may well ave consisted of porocyte processes.

Thus there are grounds for believing that the porocytes are directly inter-onnected by a perforated membrane on which the choanocytes can move and hrough which they can pass; and it seems probable that this membrane, if s existence can be established, will be the part responsible for the contractility f the internal surface. Otherwise the contractility is dependent on the ability f the collar-cells to develop tension and to cohere together.

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he Anatomy of the Infra-red Sense Organ in the Facial Pit of Pit Vipers

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With four plates (figs. 1, 2, 3, and 5)

SUMMARY

1. The histological composition of the sensory membrane in the facial pit of rattle-
akes (*Crotalus* spp.), the disposition of the nerve-trunks entering it, the fibre-size
ectrum of the nerves, and the form of the sensory endings are described.

2. Between the two layers of extremely attenuated epidermis the principal consti-
ent of the membrane is a single layer of specialized parenchyma cells with osmiophil,
ticular cytoplasm. These are not regarded as sense cells but they react strongly and
cally to degeneration of nerve-endings.

3. The axons enter through numerous trunks from three branches of the trigeminal
rve, from all sides of the membrane, providing a dense innervation. They lose their
yelin, taper to about 1μ, then expand into flattened palmate structures which bear
any branched processes terminating freely over an average area of about $1,500\mu^2$,
erlapping only slightly with adjacent units but leaving virtually no area unsupplied.
his means there are from 500 to 1,500 axons ending per mm², an estimate which agrees
th the nerve-counts. No other form of ending was found.

4. The mode of the fibre-size spectrum lies in the region $5-7\mu$ diameter.

5. A transmission spectrum of the fresh membrane shows broad absorption peaks
3 and 6μ and about 50% transmitted in other regions out to 16μ. The visible spectrum
at least 50% transmitted and probably much is lost by reflection. Strong absorption
kes place at wavelengths shorter than 490μ.

6. The anatomical adaptations of the sense organ are discussed, especially the con-
ntration of warm receptor fibres, the thinness of the membrane, the extremely
perficial position of the nerve-endings—all increasing sensitivity to caloric flux. The
erhanging margins of the pit and the richness of supply are believed to permit
rectionality of reception.

7. It is suggested that the palmate form of the ending has a significance in permit-
g several independent local sub-threshold activity generators to coexist in the
ocesses and in pooling their coincident, electrotonically spread potentials to influence
e initiation of spikes which may take place at the junction of axon and palm.

CONTENTS

INTRODUCTION

IN a recent communication the properties of the sense organ in the remark-
able facial pit of pit vipers have been analysed physiologically (Bullock
and Diecke, 1956). It is shown that this structure is highly sensitive to inter-
mediate and long infra-red radiation to or from the snake. The conclusions
are reached that the organ is specialized for the detection of solid objects of
slightly different surface temperature from background objects, that the
object can be rather small, that there is a certain degree of directional sensi-
tivity, and that the special features are not so much measured by a high
temperature sensitivity as a caloric flux sensitivity. The receptors themselves
are regarded as warm receptors, a type poorly known heretofore, functionally
and anatomically. The three sizeable nerve-branches supplying it are found
to be nearly pure populations of warm fibres.

These results, together with the unsatisfactory state of the descriptions
in the literature, have prompted us to re-examine the facial pit organ histo-
logically.

The facial pit is peculiar to and characteristic of the crotalid snakes and has
hence aroused attention for a long time. Lynn (1931) gives an account of the
early studies and lists seven theories of its function that have been proposed.
We may confine our notice here to the five workers who have undertaken
microscopical investigation. Desmoulins (1824) was apparently the first to see
the rich innervation and concluded that it was sensory, probably olfactory in
function. Leydig (1868) treated it, together with other unfamiliar structures
in lower vertebrates, in a memoir on organs of a sixth sense. He provided a
rather good description of its histology but described the nerve-fibres as
originating in 'terminal ganglion cells' in the sensory membrane in spite of
recognizing that they are trigeminal nerve-elements, presumably with cell-
bodies in the Gasserian ganglion. As we shall see, the strange form of the free
nerve-endings does in fact resemble a nerve-cell with several branching pro-
cesses and it is possible that Leydig saw some of them, only erring in thinking
he saw a nucleus. In any case he came closer than later workers for the next
80 years.

West (1900) provided a more complete account and may also have seen
some actual endings. But he also believed they were cells and identified as
nerve-terminations in sections of embryos what must have been epidermal
cells and in sections of adults what we call the parenchymatous cells of the
membrane. It is remarkable that these workers made out as much as they did,
when we recall that they had only a few, poorly fixed, mostly spirit specimens
of these snakes. Not until Lynn (1931) took up this object did a worker with

ccess to fresh material exploit this advantage. With respect to the micro-
copic structure of the membrane, however, little could be added and the
ame mistake was perpetuated concerning fifth cranial nerve-fibres with cell-
odies in the periphery. This mistake was corrected by Noble (1934) and
Noble and Schmidt (1937), who identified the cells of West and Lynn as
pidermal cells preparing for shedding and with silver impregnation described
ree nerve-endings. As we now see, they still did not see the endings. Their
mpregnations, like many of ours, were incomplete. There is also some reason
o doubt their identification of parenchyma as outer epidermis.

MATERIALS AND METHODS

Several species of rattlesnake were used, *Crotalus atrox*, *C. cerastes*, *C. ruber*,
C. horridus, *C. adamanteus*, but mostly *C. viridis*. No consistent differences
etween the species were noted in respect to the characters under study; it
s probable, from incidental observations, that there are differences in the
degree of pigmentation of the pit and especially the sensory membrane. This
is likely to have little functional significance.

The membrane at the bottom of the facial pit was studied microscopically
n its natural position by reflected and by transmitted light. It was removed
nd observed while fresh. Intravitam methylene blue staining was attempted
ut without significant success. Overfixing in 1% buffered osmium tetroxide
(Palade, 1952) was found to be useful for visualizing the disposition of the
erve-branches in the membrane and following individual fibres to the point
where myelin stops abruptly. Alcohol formaldehyde/acetic, Carnoy, Bouin,
nd the fixatives required by special silver methods were used to reveal fibre-
ndings in both whole mounts and sections. Sections were cut in low viscosity
nitrocellulose or in paraffin or double embedded and stained with Masson's
richrome stain or silver-on-the-slide methods (Holmes, 1943; Romanes,
950). A large number of whole mounts of the membrane were treated accord-
ng to these as well as the silver procedures of Palmgren (1951), Weddell and
ander (1950), and Bodian (1936), and variations of these. A small number of
hese showed nerve-fibres in various degrees of completeness and a still
maller number showed the palmate expansions beyond the end of the myelin
heath, with their rich aborization of branching processes. We believe a
easonable argument can be made that these preparations show the endings
irtually completely, but of course we have no assurance of this.

Snakes in which a nerve or two nerves had been cut were kept for various
eriods and then the pit membrane was removed and over-fixed in osmium
tetroxide to show the pattern of degeneration from that nerve.

One very large specimen yielded a membrane so large as to permit mount-
ng, fresh, in specially made adapters. The transmission spectrum between
and 16μ wavelength was recorded with a Baird Associates automatic infra-
ed spectrophotometer, and transmission through the visible into the near
ltra-violet was recorded with a Beckman DU and a Beckman IR2.

In order to estimate the fibre-size spectrum in the nerves supplying the pit,

these were fixed in Flemming's fluid (Lillie, 1948), embedded in paraffin, cut, and mounted without staining.

<div align="center">RESULTS</div>

Histological composition of the sensory membrane

A cutaway drawing of the pit showing the sensory membrane and its relation to the rest of the head is given in the physiological paper (Bullock and Diecke, 1956). Further details of the gross anatomy are provided by the earlier authors mentioned above. The portion of concern here is the thin, richly vascularized and innervated, slackly suspended, dry membrane which forms the floor of the pit. It separates the pit or outer chamber from an air-filled inner chamber which has communication with the outside through a special duct opening into the adnexa of the eye. This means that the membrane is in contact with air on both sides. Its thickness in recently-shed adult rattlesnakes is 10μ or slightly less except for local thickenings, especially where nerve-bundles lie. As other authors have shown, it is thicker in developmental stages and we believe it may be thicker in some of the other genera of the family. At 10μ it seems to have reached a kind of limit dictated by the size of the erythrocytes. Added to this thickness there will normally be a few microns of the multi-layered cornified epidermis preparing to be shed.

On both outer and inner surfaces there is a cornified epidermal layer (fig. 1). This doubtless varies in thickness and number of layers with the stage in the moult cycle; in our sections it is from 0.5 to 1.5μ, whereas in a figure given by Noble and Schmidt (1937) it is several times thicker. According to Lange (1931) the layer with which this is continuous in the ordinary epidermis should not be called a cuticle, for he believes it is a proper cellular layer and he applies the term *Oberhäutchen*. We can confirm that the shed skin of the pit consists of two cellular layers but there is also a third layer without cell outlines or nuclei which appears to be a cuticle. For all its thinness, the outer epidermis must have remarkable physical properties since there is virtually nothing else standing between the soft tissues and the dry air. It is not essentially different on the two sides of the membrane except that the inner layers are less cornified.

Under the cornified layer, since it is periodically shed, should be a germinative epidermal layer. This is easily seen in the embryo (Noble and Schmidt, 1937; Lynn, 1931), and in occasional thicker places in the adult membrane. But in the typical adult structure it is so thin that it cannot be traced continuously in our sections. Much flattened nuclei, 0.5μ thick, are encountered at long intervals applied to the underside of the superficial cuticular layer and these we believe represent the basal germinative cell-layer. This layer seems to be even more reduced on the inner surface than on the outer.

FIG. 1 (plate). Cross-sections of sensory membrane.

A, the junction of the membrane and the side wall of the facial pit; anterior chamber above, posterior chamber below; Masson stain. *C. atrox.*

B, reduced silver method; region of a bundle of myelinated fibres some distance from their endings; outer side of membrane above. *C. viridis.* Note sections through fine unmyelinated endings (arrows).

FIG. 1
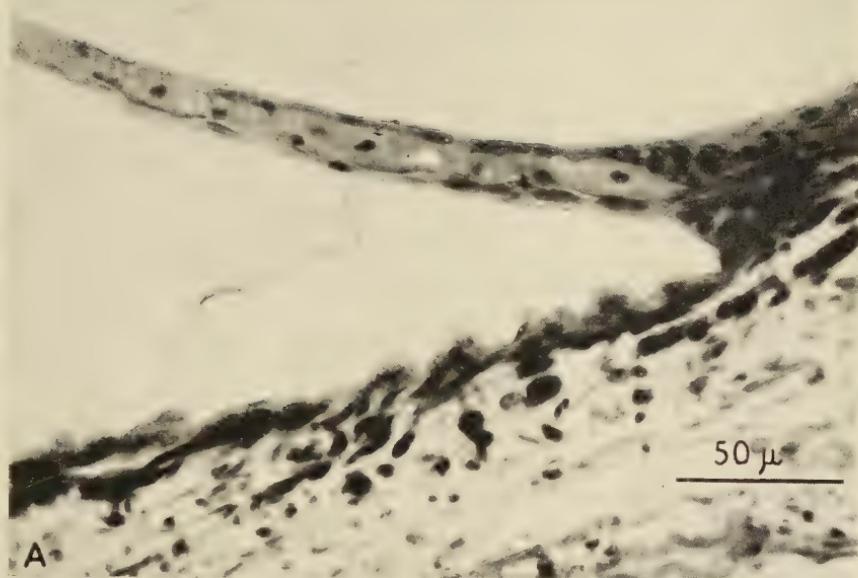
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Under the epidermal layers on both surfaces is a distinct stratum staining green with the Masson trichrome in contrast to the layers above and below it. This is generally 0·5 to 1·5 μ thick or even vanishingly thin on the outer side but 1 to 2·5 μ or even much thicker in local regions on the inner side of the membrane. We believe these layers to represent connective tissue of the dermis or corium, contrary to Noble and Schmidt (1937), who identify everything from the outer surface through the next and deepest layer as outer epidermis and thus recognize but a single connective tissue-layer, just beneath the inner epidermis. To be sure, the outer layer of connective tissue is so thin in most of the membrane that our only reason for so identifying it is its staining affinity. But the inner has discernible collagenous connective tissue fibres. Both can be followed into thicker regions where they become continuous with typical connective tissue. Fibres which appear to be collagenous connective tissue-fibres are visible in some silver impregnations of whole mounts where they reveal a sparse array of thin, straight fibres running in all directions for long distances. Both outer and inner layers of connective tissue show nuclei though in the thin parts of the membrane these are far apart and very much flattened (fig. 1).

This brings us to the middle and thickest layer which may be called the parenchyma. This is a single-cell layer, generally between the limits of 4 and 12 μ thick. The cells are quite specialized in having, unlike any others in the membrane, an abundant cytoplasm which is coarsely reticular, almost granular, and stains darkly. In Masson's stain it takes a dull red, distinct from the brighter red of the epidermis in the side walls of the pit. In osmium preparations and in some silver impregnations this cytoplasm takes up considerable colour. The cell-type is not easily identified with any in ordinary skin. It is here regarded as part of the dermis since it lies between connective tissue-layers and is cut up into lobules by the capillary bed which occupies the same level of the cross-section. The nuclei are rich in chromatin and often show two nucleoli, but are mainly distinguished from the epidermal nuclei by being commonly a little irregular in outline rather than smoothly oval as are those of the epidermis of the sensory membrane.

The vascular bed has been figured by Noble and Schmidt (1937) and we can confirm the general character of the blood-supply as described. Being in a single plane, it is easy to visualize in the living membrane in its natural position, and also in prepared whole mounts. The richness of the supply varies considerably and can be suggested by stating that the maximum distance from any point to the nearest capillary is between limits of about 30 and 60 μ.

Aside from the nerve-supply, the only other element in the membrane is a scattering of chromatophores. In the species of *Crotalus* examined melanophores are very few; they are much more abundant in *Agkistrodon*. However, in silver whole mounts we often see extensive patches of what appear to be pigmentless chromatophores which have taken up a granular metallic impregnation. These have occasionally been recognized in cross-sections as very

thin, dark-staining masses immediately beneath the outer-most epidermal layer. They may send processes toward both surfaces.

Nerve-endings in the sensory membrane

Fig. 2 shows the aspect of the endings as revealed by our best silver-stained whole mounts of the membrane. The axis cylinder, having tapered down to 1 or 2μ and lost its myelin sheath, suddenly expands into a very flat, broad, palmate structure from which 3 to 7, usually 5 or 6 processes spring to branch repeatedly and end as exceedingly fine, free endings. The thinness of the membrane is very favourable for working out the details of these remarkable nerve-endings because it permits whole mounts in which no concern is necessary over processes that go out of the plane of a section, or over apparent endings which may really be created by a knife; and the confinement to a plane of not more than 8μ depth facilitates following the branches. In the drawing every twig is taken from a camera lucida tracing.

The palms exhibit a well-developed reticulum of neurofibrils in reduced silver stain, and these can be followed into the larger processes and for some tens of microns up the axon. The aspect of the palm strongly resembles that of a multipolar neurone with several large dendrites and one axon, but without a nucleus. The branching of the processes is profuse, not dichotomous or regular or with many thorn-like offshoots, but irregular, often at small angles, often quite unequal and often multiple at a given point. Branches characteristically sweep back in an arc toward the palm but at a more superficial plane.

No palm is traceable to an axon which bears another palm. No branch or process is traceable with certainty into two palms, i.e. there is no anastomosis. Processes not uncommonly swell up into minor palms where they branch but this is so obvious that it is not difficult to establish a one-to-one relation between axons and palms. No axon has been seen to end in any other way than as one of these palmate expansions with processes. The palms vary in size but over a limited range of approximately 2 to 4μ across the widest waist between processes, and 20 to 30μ in long dimension between the base of the axon and that of the farthest process. We have not discerned classes based on size or other features and must conclude that, within a range of variation, we have to deal with a single morphological type of sensory ending.

The area of ramification of the terminals of one axon generally does not extensively cross capillaries; it commonly falls entirely inside one mesh of the capillary net, but there is usually more than one palm per mesh. The terminations of the processes seem typically to conform to the outlines of parenchyma cells where these are visible, as at the edge of a lobule of them. The terminations are profuse and end among the parenchyma cells, as well as superficial

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Fig. 2 (plate). Silver stained whole mounts of facial pit membrane showing sensory endings. *C. viridis.*
   A, a drawing of an actual field.
   B, a photomicrograph of a similar field.

50μ
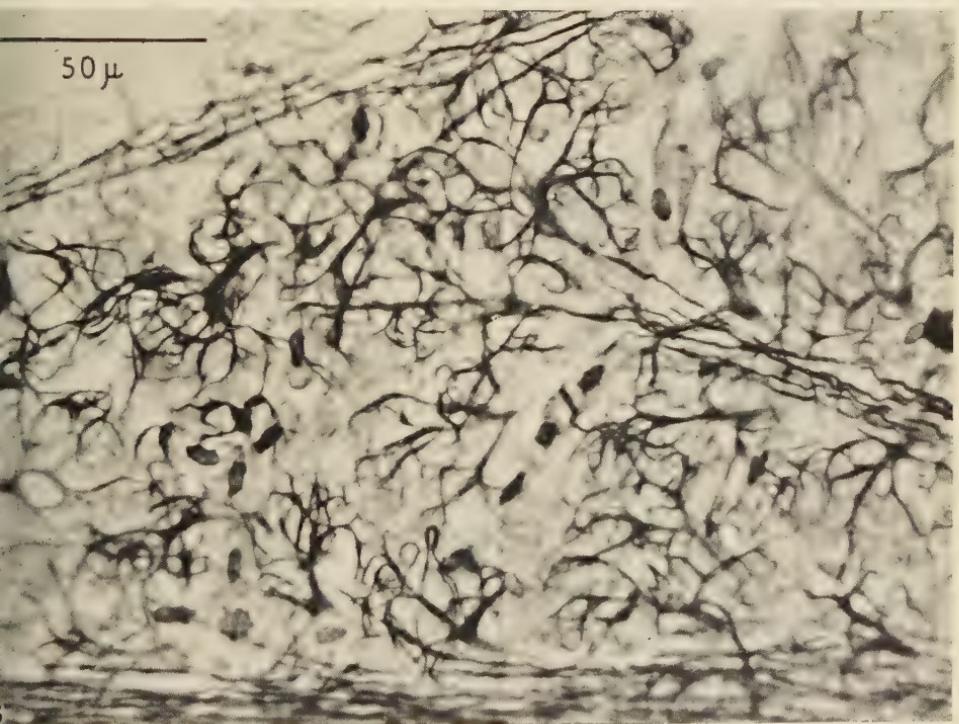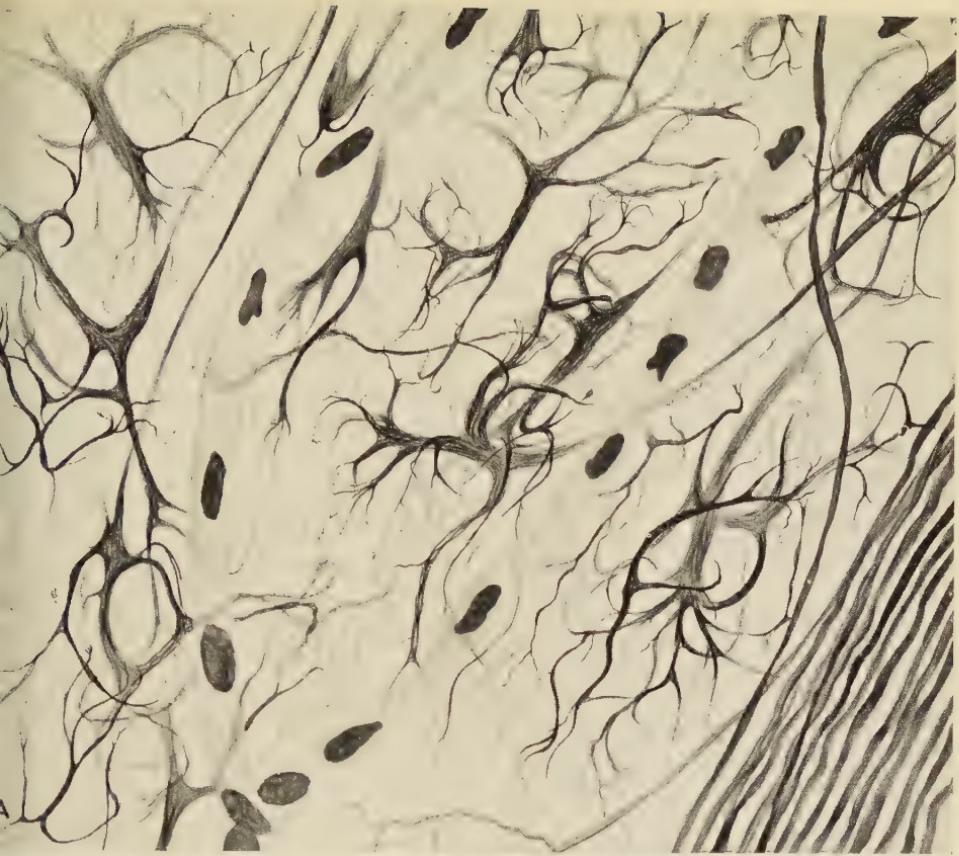
FIG. 2

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o them and, in smaller numbers, in the layers below them. Whether there are nerve-terminals among the epithelial cells we cannot say, but certainly most of them are not in that layer which is so exceedingly thin, while the terminals are found through 6 or 7μ of thickness of the membrane. The palms lie at the same depth as the capillaries, generally about 5 to 7μ below the outer surface, but are superficial to the capillaries when they cross them. Over the large nerve-trunks the palms are superficial to these. The palms are only about $2\cdot5\mu$ thick at maximum. The processes mostly proceed toward terminations in a more superficial plane, a large part of them terminating 4 to 5μ above the palms, therefore very close to the surface. A few processes pass downwards 2 to 3μ towards the inner surface.

There is not a great deal of overlap of area supplied by adjacent palms. Some interdigitation of terminal branches is seen in every field in good impregnations, but it is, broadly speaking, not very extensive and most of the area of branching of the processes of one palm is not invaded by others. This area is not simple in outline but rather is indented and produced. It is therefore not easy to trace and hence to measure. An approximation of the typical area within which the branches of one axon ramify would lie between 1,000 and $2,000\mu^2$, but these are not outer limits. Diameters of 30 and of 50μ are not rare. An estimate of the average area, not including overlap, can be obtained by counting the number of palms in a microscopic field of known size. In 18 fields chosen at random and including various parts of the membrane, an average of 520 palms per mm^2 or $1,920\mu^2$ per palm was found. This figure can be regarded as slightly overestimating the area per palm and underestimating the number of palms since it is difficult to allow for the palms which are only partly in the measured field.

Disposition of nerve-trunks entering the membrane

The pit membrane is supplied by three divisions of the Vth cranial nerve, as has been fully described by Lynn (1931). The smallest supply comes from the ophthalmic division which approaches the pit from above and divides into a small number of branches which enter the dorsal and posterior quadrant of the membrane. The deep branch of the supramaxillary division which approaches from below medially through the floor of the orbit and divides into 5 or 6 trunks, entering the anterior dorsal side of the membrane, is sometimes the largest nerve. The superficial branch of the supramaxillary division which approaches from behind and divides into 5 or 6 trunks entering the posterior and ventral aspects is perhaps more commonly the largest. Each of these nerves sends fibres also to the skin of the head, lips or roof of the mouth, according to Lynn, but Bullock and Diecke found exceedingly little evidence of action potentials in the last two nerves in response to tactile stimulation of the skin outside the pit and the activity characteristic of them was completely silenced by a drop of cool water placed in the pit. Our own gross dissections and cleared heads with the nerves stained with haematoxylin revealed very few twigs from the three nerves going elsewhere than into the pit membrane.

Certainly we can agree with Lynn's statement that these nerves are over-whelmingly concerned with this organ.

The nerve-trunks as they enter the membrane are of quite unequal size, 10 or 12 in number and rather evenly distributed around its periphery (fig. 3, A). They immediately begin to splay out, losing size fibre by fibre and sending off a small number of branches. The larger trunks are discernible almost to the centre of the membrane. They travel on the inner side of the cross-section, between the parenchymatous layer and the inner epidermis and under the capillaries.

As they fan out, the fibres travel short distances and then lose their myelin sheaths (fig. 3, B). Shortly thereafter they expand into the terminal palms but an unmyelinated segment of about 20 to 50 μ intervenes. The unmyelinated segments come to lie superficially to the capillaries.

The fibres from adjacent trunks do not considerably interdigitate but supply areas of the membrane with sharp, non-overlapping boundaries. This can be seen by tracing from a silver-stained whole mount the palms in the region of a boundary and noting the directions of the axons. These fall cleanly into two groups with a wavy line between the palms belonging to the two. It can be seen most strikingly in the preparations where one nerve has degenerated (see p. 227).

Composition of the afferent nerves

The fibre-size spectrum in a typical case is given in fig. 4. There appeared to be no significant difference between specimens examined of *Crotalus atrox*, *C. horridus*, and *C. viridis* of snout-vent length from 701 to 965 mm. There may be some tapering between a level 10–15 mm from the membrane and the sizes close to the membrane, but if so, it is very slight as reflected in the mode. In three ophthalmic nerves the broad mode lay between 4 and 6 μ (uncorrected diameter of myelinated fibres), in one nerve between 3 and 5·5; the largest axons in these small nerves were usually between 7 and 8 μ. In 5 deep branches of the supramaxillary, the modes lay from 3·5–7·5 to 5–9·5 and the largest fibres reached 17 μ, although only a few per cent. of the fibres are above 9 μ. Four superficial branches of the supramaxillary division lay in the same range and, for comparison, three mandibular nerves supplying ordinary skin gave curves which overlapped completely with these.

In the pit membrane shortly before the myelin sheath stops, the diameters appear to have fallen somewhat but are still typically 3 to 5 μ.

We have not attempted to estimate the population of unmyelinated fibres in these nerves. In the silver-stained preparations of the membrane there do

FIG. 3 (plate). Osmium preparations (whole mounts) of facial pit membrane showing myelinated nerve fibres. *C. ruber.*

A, the whole membrane. Note capillary bed.

B, higher power to show individual fibres. Note myelin sheaths abruptly ending; this is a few tens of microns from the palmate-ending.
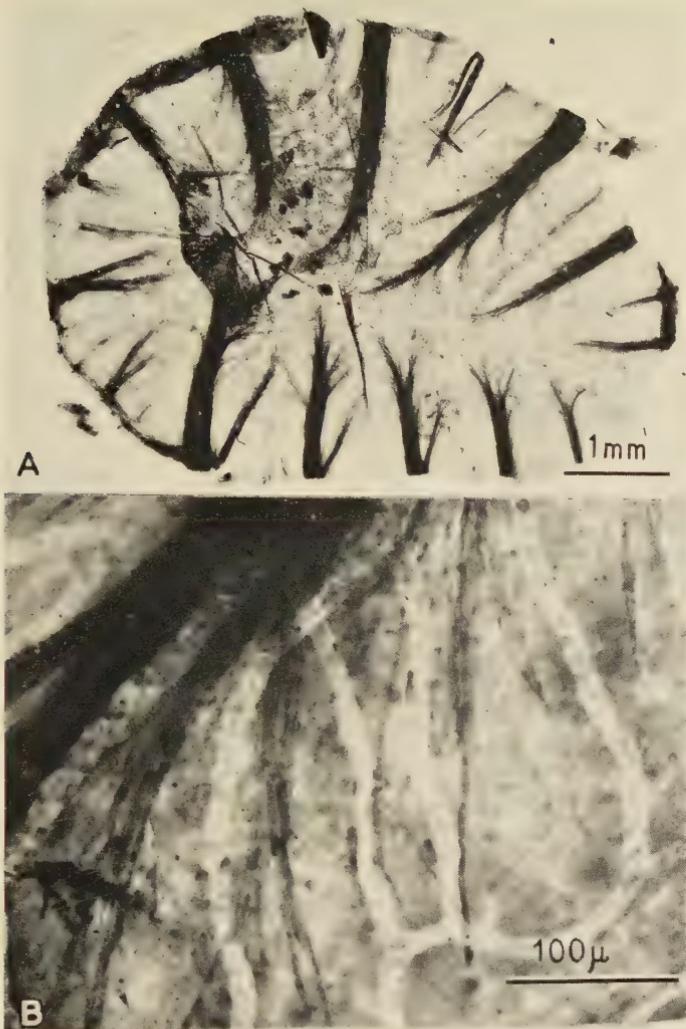
FIG. 3

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not appear to be stained fibres of much smaller diameter or of a different form of termination from the myelinated fibres described already.

The number of fibres in the cross-sections of the nerves is of interest as an independent means of calculating the total nerve-supply to the sense organ.
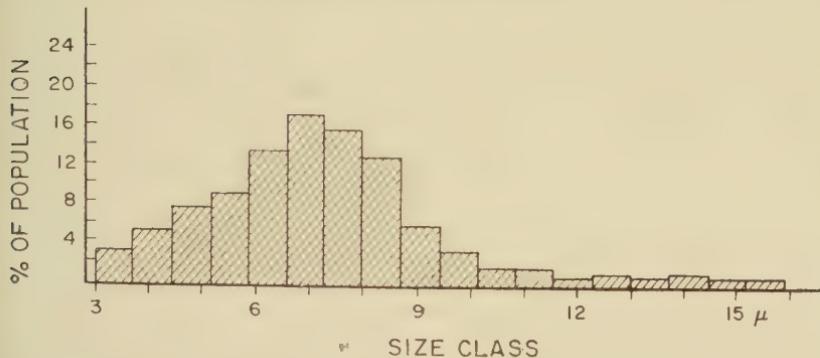
Fig. 4. Composition of an afferent nerve. The superficial branch of the supramaxillary division of the trigeminal nerve in a specimen of *C. viridis*. The graph shows the diameters of 295 fibres from a random assortment of fields.

It should give a reliable maximum figure, although the figure will be too high by an unknown amount due to the component destined to supply the ordinary skin of the anterior part of the head. Counts of representative aliquot parts of each cross-section, corrected to the total cross-section in a 965-mm specimen of *C. horridus*, gave 815 myelinated fibres for the whole ophthalmic nerve, taken a few millimetres proximal to the pit, 2,921 fibres for the superficial branch of the supramaxillary, and 3,538 for the deep branch, a total of 7,274. In three other snakes totals of 8,254, 6,626, and 7,410 were obtained. Dividing these figures by the approximate areas of the membranes in each case gives 586, 825, 839, and 1,950 fibres per mm^2. The estimates of areas are particularly subject to error, being made on the excised, concave membrane, so that the real scatter may not be as great as is suggested by these figures.

Degeneration after cutting one or more of the nerves

Five specimens which had had one or two of the nerves cut 1 cm or more from the pit were kept for a few weeks to allow degeneration of the severed axons and then prepared by osmic fixation and staining of the sensory membrane as a whole mount. A period of 2–3 weeks appeared optimal at room temperature for a clear distinction between degenerating fibres and normal ones without too much of the myelin debris being already removed by phagocytes—a process which is beautifully shown in these whole mounts in later stages.

By this means it is possible to map the area of supply of the separate nerves. The nerve-fibre counts in the cross-sections of the nerves have already indicated that the relative contributions of the several nerves vary somewhat. We

cannot be sure, therefore, that in the few cases mapped a typical picture of the distributions was obtained. The ophthalmic nerve has not been cut alone but after cutting the other two a very small segment of the membrane, less than one-sixth of its area, remains supplied by undegenerate fibres. After cutting the superficial nerve, a segment of about 60% of the area shows degenerate fibres. The deep nerve, by difference, supplies about half the membrane. These segments do not overlap in the slightest. We have not attempted to preserve accurately the orientation of these segments relative to the body axes.

An astonishing feature of these preparations is that, besides the contrast between normal and degenerating nerve-fibres in the two parts of the membrane, there was found in a few cases a striking contrast between the two regions in the staining affinity of the parenchyma cells (fig. 5, A, B). This means that the whole background in the osmium preparation (cleared whole mount) is either dark (in the normal part of the membrane), or pale (in the part innervated by the now degenerating fibres). Moreover, the separation between these two conditions is sharp. The line is easily followed under the high powers of the microscope because each cell in the region of the boundary is either dark or pale, i.e. they do not intergrade and the line is unbroken: dark cells are not found on the pale side of the boundary or conversely. Curiously, the line does not consistently follow capillaries but it sometimes does. This means that it goes through the middle of the lobules of parenchyma defined by the vascular bed and, therefore, presumably that given nerve-fibres actually do send terminal branches across capillaries more often than was indicated above from the silver preparations. We have not attempted to go further into the pathological histology to inquire what is the nature of the change in the parenchyma cells. It would seem of considerable interest as a dramatic example of the dependence of peripheral, non-nervous cells for some trophic requirement upon the intactness of the nerve-endings, in this case sensory endings.

Transmission spectrum of the membrane

Physiological data indicated that the action spectrum for adequate stimulation of the receptors by radiant energy lies essentially between 1·5 and 15 μ or more of wavelength, i.e. in the medium and long infra-red, and that visible light is virtually ineffective (Bullock and Diecke, 1956). This action spectrum must be entirely contained in, i.e. must lie under, the absorption spectrum of the membrane, although they may not coincide. That is, only the energy absorbed can have an effect, although not all energy absorbed need contribute to the stimulation of the receptors. But, according to the hypothesis reached

Fig. 5 (plate). A and B, normal and denervated regions of membrane seen in an osmium preparation (whole mount). *C. atrox.* The superficial branch of the supramaxillary nerve was cut 23 days previously. Nerve-fibres degenerate and parenchyma cells change staining quality in the area of membrane supplied by this nerve. Note absence of overlap with normal area. c, a common cell-type in the membrane, identified tentatively as pigmentless chromatophores. *C. viridis.* Silver-stained whole mount.
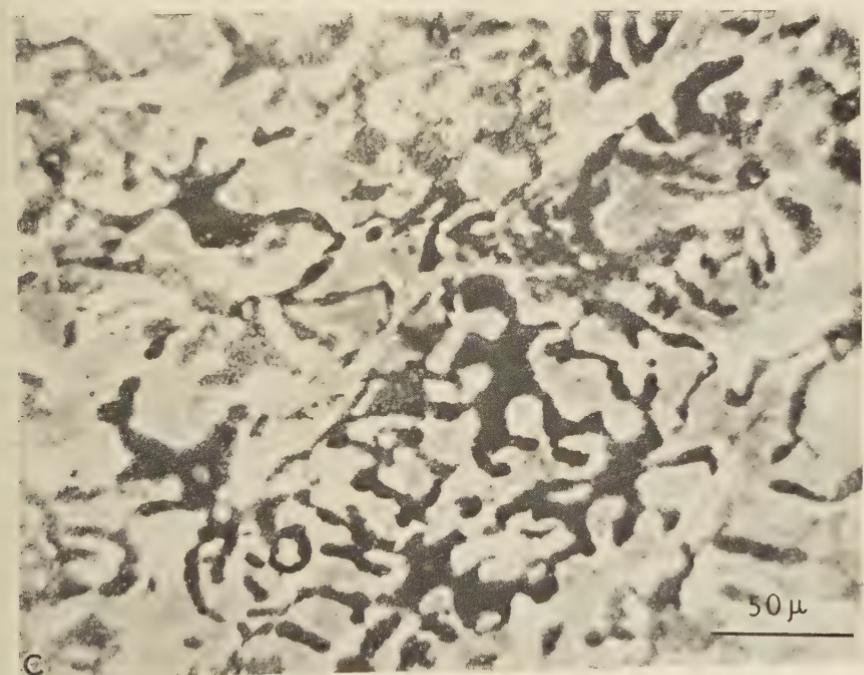
FIG. 5

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amely that the receptors are responding to the temperature changes of the
ssue resulting from absorption of radiant energy, the absorption spectrum
n be expected to coincide with the action spectrum since the heating effect
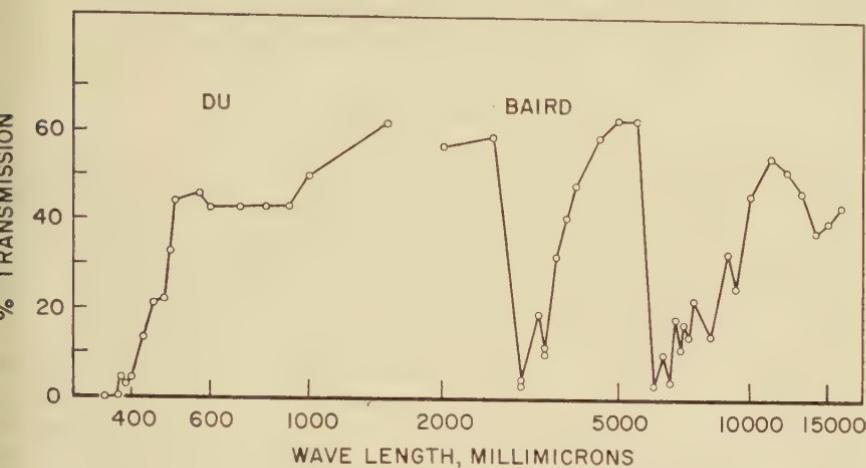
FIG. 6. Transmission spectrum of fresh membrane. *C. adamanteus.*

ill not discriminate between different wavelengths absorbed. A lack of
oincidence could be caused by the absorbing structure being unable to heat
e receptors, because of distance between them or loss of heat by transport,
g. in erythrocytes.

One specimen of *Crotalus adamanteus* became available which was so large
at its two pit-membranes together covered a window of usable dimensions
r spectrophotometers, 2 by 15 mm. The freshly dissected membranes were
ashed free of surface blood, dried for 10 min in a P_2O_5 desiccator under
rtial vacuum, and mounted by Scotch tape while being held flat with a glass
ide. The membrane is naturally curved in both directions, like an orange
eel, and cannot be perfectly flattened. Although the light beam consists of
arallel rays normal to the plane of the membrane, there is necessarily some
ss of light due to reflection and scattering, and this is of unknown amount.
or the Baird Associates IR spectrophotometer (NaCl prism), a single
embrane in a window 2 by 7·5 mm was used. The results are shown in fig. 6.
he main features of interest are the deep absorption maxima at 3μ and at 6μ,
e gradual rise in transmission from 6·6 to 10μ, and the rather high (approxi-
ately 50%) transmission in the long infra-red out to at least 16μ. The latter
not usually associated with tissue or organic substances, especially with high
ater-content, and must be put down to the thinness of the membrane.
nce the long infra-red is the region of most effective stimuli, these being
om objects of a temperature only a few degrees different from the snakes,
is finding indicates a large percentage inefficiency.

The Beckman DU spectrophotometer showed no large absorption maxima
the near infra-red or visible regions but at wavelengths shorter than 490μ

absorption rapidly increased and was virtually complete at 380μ. The trans mission through the visible was not high (between 40 and 50%), so that eithe there were sizeable losses by scatter and reflection or there is a discrepanc between the absorption and the action spectrum, since visible light was in effective in stimulating unless at very high intensity.

<div align="center">DISCUSSION</div>

The anatomical adaptations of the sense organ

It was pointed out by Bullock and Diecke (1956) that the physiologica specializations of this organ were not profound. Sensitivity is much highe than in other temperature receptors directly studied by nerve impulse record ing, but not higher than that calculated for man. The steady state sensitivit found in the other known temperature receptors is not present here. Th unusual forms of response to strong stimuli are not necessarily adaptation Most of the adaptations of the organ are anatomical.

The primary one of these is the concentration of warm receptor element into a pure population of that modality. The high density of these element means that full advantage can be taken of the phenomenon of central summa tion (in which the threshold is lowered by simultaneous stimulation of man receptors), even though a very small area of the skin is stimulated. This mean that the stimulation flux (calories per cm² per sec) is small. Central summatio is the phenomenon probably mainly responsible for behavioural threshol assuring as it does that weak stimuli which give unreliable signals in un receptors give quite reliable signals in a group of parallel channels.

The thinness of the sensory membrane probably confers a higher sensitivit to caloric flux in a certain range of stimulus durations. If we separate fror thinness the closeness of the nerve-endings to the surface, that is, compare thick membrane with endings equally close to the surface, then thinness wi permit a given flux to warm the receptors more, provided that the stimulu is not allowed to act indefinitely and provided that it is allowed to act longe than a very brief period. In the case of a very brief stimulus, the advantag of thinness decreases because there is proportionally less loss of heat t the depths in the thick membrane than there is for longer stimuli. In th case of very long stimuli warming approaches a steady state and, unles there is a large sink in the thick membrane into which heat flows, the will be little advantage in a thin membrane. The range of periods f which the membrane as we see it offers an advantage cannot be accuratel calculated without more knowledge of the loss of heat to the blood, b apparently it extends from a second or less to many seconds. This is base on the consideration that in a thin membrane and neglecting losses reradiation and blood-flow, the change in temperature for a given flux proportional to the time, while in a thick structure which conducts he past the receptors, the change is proportional to the square root of the tin (see Bullock and Diecke, 1956).

Perhaps more important than the thickness of the membrane is the super-
ficial location of the receptors. We could not give accurate measurements of
the depth of the endings, but the average depth is probably no more than 5μ
and possibly only 2μ from the outer surface. This not only increases the
promptness of the response and the ability to detect flickering and brief
stimuli, but situates the detector at the most favourable place in the tempera-
ture gradient. Actually we can say, from the transmission spectrum, that the
adaptation has gone about as far as it is worth going, for the layer between the
nerve-endings and the surface is already so thin that it is absorbing something
less than half of the energy in the wavelengths chiefly available.

We need not discuss here the special features of the posterior chamber, its
outlet and sphincter, the location on the head, the angle of view, the circula-
tion, or the chromatophores (fig. 5, c). But one further feature of the anatomy
of the accessories deserves mention. This is that the pit characteristically does
not taper inward but actually overhangs, i.e. the mouth is smaller than the
membrane at the bottom. This is obvious to the eye and in a small series of
randomly chosen specimens of four species, the diameter of the pit opening
of 10 pits was from 35% to 50% of that of the membrane. The consequence
of this, together with the small depth of the pit, is that most radiating objects
unless very large or very close will not illuminate the whole sensory membrane
but will cast shadows of the pit margin. This confers the possibility of deriving
information from the sense organ about the direction of small objects or of
edges of large objects, if the resolution of the sense organ is sufficient. We have
already seen that the innervation is rich. There are between 500 and 1,500
axons ending per mm². This compares with about 800 optic nerve fibres per
mm² of the retina in man, averaging an estimate of 800,000 fibres into an
estimate of 1,000 mm² of retina. The membrane provides enough absolute
area and hence nerve-fibres to resolve even fairly poor shadow margins: in
snakes between 350 and 1,000 mm snout-vent length the membrane diameters
fall near a line connecting 2 and 4·4 mm, corresponding to areas somewhat
over 3 and 15 mm² (since the surface is concave). We may conclude that the
central nervous system receives adequate information to analyse directionality
with a degree of usefulness, especially if the snake scans or if the object is
moving.

A final point should be raised concerning the nature of the parenchyma
cells of the sensory membrane. These are the only non-nervous cells of the
sense organ which are specialized, except for the attenuation of the epithelial
and connective tissue-cells. They are not readily identified with any cell-type
of ordinary skin. They are polygonal, about 6μ thick, with an osmiophil
cytoplasm of uniform, dense, reticular structure. Most significantly, they
react with a change in staining quality to loss of the nerve-endings and evi-
dently depend on the particular ending intimately located about them; for
there is no grading off of the reaction from the boundary between normal and
denervated regions (compare Hillarp, 1946). These facts might lead in terms
of classical histology to regarding the cells as sense cells. We do not believe

R

they should be so considered unless evidence is obtained that they actuall[
respond to the normal environmental stimulus and mediate the initiation o[
nerve discharge. In the physiological study Bullock and Diecke were unabl[
to obtain such evidence, although they could not eliminate this possibility[
If, as we suppose, the parenchyma are not sense cells, they very probably d[
have some essential accessory function in connexion with the transducing o[
temperature change into neuronal activation.

Comparison with other reptilian receptors

It has been suggested in the literature repeatedly that there may be a[
analogy between the facial pits of pit vipers and the labial pits of some python[
and boas. No other organ appears to resemble these in structure or functio[
as far as is known. The labial pits are simple depressions, without an inne[
chamber and hence with a floor receiving the nerve-supply but no membran[
(Noble and Schmidt, 1937). Behavioural experiments clearly suggest a func[
tion as receiver of warm radiation, like the facial pits of crotalids, although th[
authors have apparently not realized this and speak of air temperature[
measured by a mercury thermometer (Noble and Schmidt, 1937) or air move[
ment resulting from the local heating of the air (Ros, 1935).

As far as we are aware the facial pits are not similar in anatomy to any othe[
temperature receptors among animals, or indeed to receptors of any modality[
The nerve-endings themselves seem also to be unique, in the form of th[
terminal palmate expansion. It does correspond with the conclusions o[
Weddell and his collaborators that temperature-endings should be fre[
nerve-endings and not necessarily corpuscular. Free nerve-endings have bee[
described in reptilian skin (see Boeke, pp. 859–66), but none which give an[
special suggestion of being forerunners of these. Bullock and Diecke (1956[
could not find evidence of any temperature reception resembling that con[
centrated in the pit, on searching through ordinary skin-nerves of rattlesnake[
and even the homologous branches of the trigeminal in non-crotalid snake[

Possible significance of the palmate-ending

If, as we suppose, the receptors are detecting and transducing small tem[
perature changes in the tissue, there is no special significance in the spatia[
aspect of the ramification of each axonal-ending, that is there is no particula[
advantage in covering a wide territory as there would be if interceptin[
photons or mechanical deformation were their function. But we may sugges[
that the development of an extensive surface, subdivided into quasi-inde[
pendent regions, enhances the probability of firing to a small change. This i[
based on the supposition that these fine processes are prone to autorhythmi[
sub-threshold, graded changes of state which do not propagate but sprea[
decrementally, not interfering with each other but summating so that at som[
critical point, perhaps at the emergence of the axon from the palm, all or non[
impulses are initiated whenever some sharp threshold is exceeded. We woul[
visualize the several processes of the palm each undergoing independentl[

ctuations in state, including membrane potential, and these may be quite ythmic. As they happen to summate and therefore to spread farther they uld from time to time trigger impulses in the axon. This would explain, as as it goes, the non-rhythmic spontaneous background discharge. It also akes it possible to invoke the most exquisitely sensitive mechanism of nute changes in the potential gradient between one part of the neurone rface and another, recently shown by Terzuolo and Bullock (1956), to alter e frequency of firing of already active neurones. This hypothesis of the tivity of sensory processes is not uniquely applicable to the present receptors t seems reasonable for many branching terminations of small diameter, pecially those with a spontaneous or steady state discharge. It places afferent onal terminations in a class with dendrites, in not supporting impulses or opagating a disturbance toward the axon, but influencing the initiation of pulses in the axon by small changes in potential gradient along the neuronal embrane (compare Fessard, 1956).

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he Formation of the Secretory Granules in the Liver-cells of the Slug, *Anadenus altivagus*

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SUMMARY

The process of formation of secretory granules has been studied in the liver-cells of he slug, *Anadenus altivagus*, in fixed preparations. The following conclusions have een drawn.

1. The secretory granules, which are non-lipid in nature, are formed by direct ansformation of the lipid granules. The lipid granules assume a duplex structure uring this transformation.

2. The lipid granules, which are densely packed at the basal end of the cells, seem o arise from the tip-granules of the mitochondria.

3. The mitochondria are fibrillar and each of them shows one prominent granule at ach tip. The mitochondria as well as their tip-granules are stainable with iron haema-xylin after Regaud, whereas the lipid granules are not.

INTRODUCTION

I N a previous publication on the liver-cells of the slug, *Anadenus altivagus* (Rishi, 1956), I have described the morphological aspect of the Golgi odies and their transformation into the secretory granules as seen under the hase-contrast microscope. In that paper the probable origin of the Golgi ranules of dark contrast (*Praesubstanz* of Hirsch, 1939) from the tip-granules f the fibrillar mitochondria was described. These Golgi granules were shown o form the Golgi spheroids, which ultimately were transformed into the ecretory granules.

In the present communication I have examined the same phenomenon in he liver-cells of the same species of slug, in fixed material. Special care has een taken to employ the techniques of known reactions. Techniques in-olving excessive precipitation of metallic silver or osmium have been care-ully avoided.

MATERIAL AND TECHNIQUE

The material for the present study of the liver-cells of the slug, *Anadenus ltivagus* Theobald, was fixed during the months of August and September 955 at Simla, Panjab, India. Small pieces of the liver were removed from the ving animals and placed directly in various fixatives. The fixatives employed ere Helly, Regaud, Aoyama, formaldehyde-saline, Altmann, and Champy. he material was also postchromed in a saturated solution of potassium ichromate at 37° C for 48 h after Helly, Aoyama, and formaldehyde-saline. he paraffin method of embedding was employed. Sections were cut at 5 μ.

The sections of the postchromed material fixed in Helly, Regaud, Aoyama and formaldehyde-saline were coloured with Sudan black B according to the technique described by Thomas (1948). The best results were obtained from the Regaud-fixed material.

The material fixed in Altmann and Champy was post-osmicated in a 2% solution of osmium tetroxide at 37° C for 48 h. Unstained preparations of Altmann material were also studied. The silver nitrate technique of Aoyama was also tried, but it introduced serious artifacts in the tissue.

Some sections of the Regaud-fixed material were also stained with 0·5% iron haematoxylin.

Some of the slides fixed in Regaud were first coloured with Sudan black, and the position of particular cells noted. The Sudan black was then extracted in 70% alcohol and the slides were stained with 0·5% iron haematoxylin to elucidate the relationship between the sudanophil granules (Golgi granules) and the mitochondria.

OBSERVATIONS

The cells of the liver of *Anadenus*, in the earliest secretory phase, show a large number of sudanophil granules filling up the whole of the nuclear end of the cell, which is on the side opposite to the lumen of the alveolus. These granules are very closely packed in this region. It also appears that some diffuse sudanophil material is present between the granules. These sudanophil granules correspond to the 'Golgi' granules of the author (Rishi, 1956). Besides these there is no indication of the presence of any secretory granules in such cells, although a few duplex spheroids (appearing as sudanophil rings or crescents in optical section), with a sudanophil rim and a sudanophobic sphere, may be seen towards the lumen end of the cell (fig. 1, A).

If such cells are stained with iron haematoxylin after the extraction of the Sudan black, one can make out a large number of fine mitochondrial fibrillae, each fibrilla bearing one deeply staining granule at each tip (fig. 1, B). Some of the mitochondrial fibrillae, however, do not show these granules. In addition to the tip-granules of the mitochondria there are other small, deeply staining granules present in this region. These are comparable to the Golgi pre-substance of the author (Rishi, 1956). The mass of the sudanophil granules does not take up haematoxylin at all. The cytoplasm of this region, however, stains slightly more deeply with haematoxylin than the cytoplasm of the lumen end.

With further progress in the secretory activity of the cell, the number of sudanophil granules increases in the basal end of the cell (fig. 1, C). It also appears that these granules now start spreading towards the lumen end of the cell. In addition to these solid sudanophil granules, one can now make out a few spheroids of varying sizes, appearing as crescents or rings in optical sections, in the cytoplasm of the lumen end of the cell. Among the spheroids can also be seen some solid sudanophil bodies, representing the intermediate stages between the spheroids and minute granules.

With advancement in the process of secretion the sudanophil granules shift further towards the lumen end of the cell and become transformed into the spheroids of duplex structure, with a completely sudanophobe 'internum' or medulla surrounded by a complete or incomplete sudanophil sheath (fig. , D). The number of spheroids seems to be inversely proportional to that of
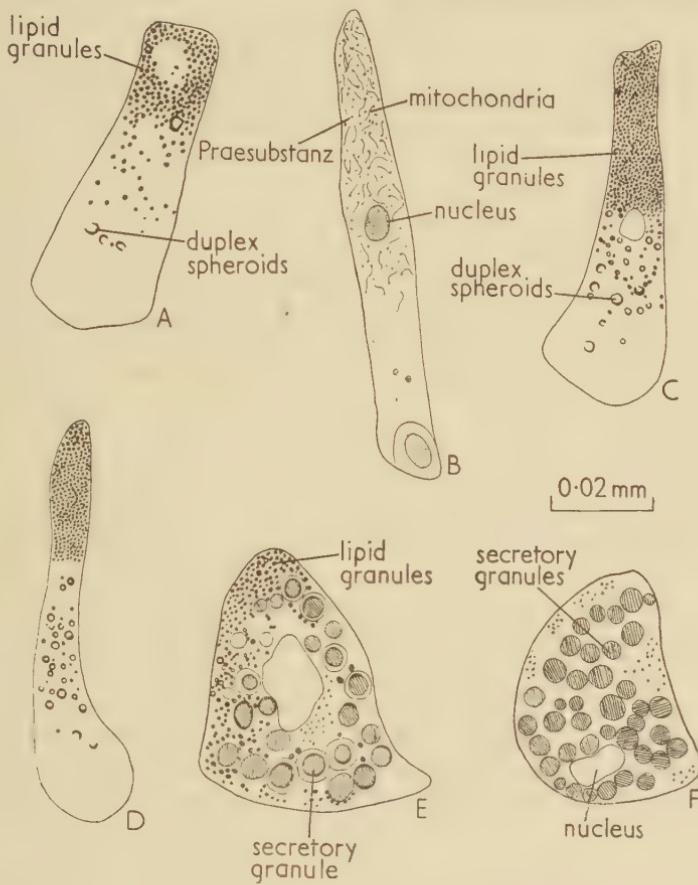
FIG. I. Camera lucida drawings of the liver-cells of the slug, *Anadenus altivagus*, fixed in Regaud's fluid. A and c–F, coloured with Sudan black. B, the Sudan black has been extracted and the section stained with iron haematoxylin.

the sudanophil granules. The latter seem to be continuously drifting towards the lumen end of the cell and becoming differentiated into the spheroids.

The duplex spheroids in the lumen end of the cell become larger and larger and their sudanophobe medulla seems to grow at the expense of the sudanophil cortex, which appears to become attenuated progressively under the pressure of the medullary growth, till ultimately a completely sudanophobe secretory granule is formed. The cell in this stage still exhibits a marked polarity in the distribution of its contents; the small sudanophil granules

occupying one end of the cell, while the secretory granules and the growing spheroids occupy the other end, which is invariably the lumen end (fig. 1, E) The amount of secretory granules continues to increase in the cell with a corresponding decrease in the amount of the sudanophil material. Ultimately the cell becomes filled up with the secretory granules, among which some sudanophil granules may be dispersed (fig. 1, F).

Neither the sudanophil granules, nor the duplex spheroids, nor again the secretory granules take up any haematoxylin after Regaud fixation.

Osmium tetroxide after Altmann blackens the sudanophil granules and the rim of the spheroids but leaves the secretory granules untouched. An exactly similar picture is presented by Altmann preparations that have not been post-osmicated.

The fully formed granules, and even the larger immature secretory ones generally appear to be surrounded by a clear space, giving the appearance of a granule within a vacuole. But since I have never seen such a space encircling the secretory granule in the living material studied under the phase-contrast microscope (Rishi, 1956), these spaces are presumably artifacts produced by shrinkage on fixation.

DISCUSSION

The present study not only confirms my earlier observations (Rishi, 1956), but also elucidates the following points.

1. The secretory granules, as well as the grey medulla of the duplex spheroids, are non-lipid in nature as they do not take up Sudan black in postchromed Helly, Regaud, or formaldehyde-saline material. The secretory granules are also completely osmiophobe and chromophobe in stained and unstained chrome-osmium preparations.

2. The dark sheaths of the duplex spheroids as well as the solid granules are lipid in nature, since they take up Sudan black intensely and are also osmiophil in unstained Altmann preparations. It seems that the sudanophil and osmiophil sheaths of the duplex spheroids undergo some chemical change and become transformed into the sudanophobe and osmiophobe secretory material.

An almost similar process in the formation of a secretory product has been described by Bhatia (1945) in the oil-gland of the common Indian duck.

Many other authors also have described the elaboration of the secretory granules in the medulla of the 'Golgi bodies'. Hirsch (1939) has shown that the secretion is differentiated in the internum of the Golgi system (duplex spheroids). Similarly Hsu (1947, 1948) has shown that the secretory droplets in the mid-gut epithelium and salivary glands of the larvae of *Drosophila melanogaster* are elaborated in the 'Golgi bodies'. According to this author, as the individual 'Golgi granule' increases in size, a light area (secretion droplet) appears in it, forming what he calls the 'Golgi-material-and-secretion complex'. Duthie (1934), working on the Harderian gland of the rat, showed that the secretory granules arise in the vacuole-like medulla of the 'Golgi apparatus',

which is surrounded by an osmiophil crescentic cortex. The granule later on fills the medulla completely.

Worley and Worley (1943) also describe a similar origin of the fat and the protein in the developing veliger larva of the tectibranch mollusc, *Navanax inermis.*

Thomas (1948) showed that the smallest secretory granules in the sympathetic neurones appear to be formed within the lipoidal pellicle of a single 'Golgi system', for it can be shown that they are at first completely covered with a sudanophil sheath. He homologizes the smallest osmiophil and sudanophil bodies to the *Praesubstanz* of Hirsch (1939).

Similarly, Nath in a number of publications since 1924 has been advocating the direct origin of fatty yolk from the 'Golgi vesicles' of duplex structure in many forms of oogenesis. For a full bibliography of the subject reference may be made to Nath (1957).

Some authors believe that chromophil or dark sheath surrounding the developing secretory granule breaks itself loose from the fully formed secretory granule, assumes the form of the 'Golgi granules', and restarts the process of secretion. This view does not seem to be applicable in the present case, because, as has been pointed out earlier, the lipoidal sheath of the duplex spheroids seems to disappear during the formation of the secretory granules, exactly as described by Nath (1957). I myself have already described how the 'Golgi granules' differentiate from the granular 'Golgi pre-substance', which in turn seems to arise as tip-granules of the fibrillar mitochondria (Rishi, 1956). A similar view on the origin of the Golgi bodies (lipoidal bodies) has been expressed by Hirsch (1939).

In Sudan black preparations of these cells it is not possible to differentiate any mitochondrial material or pre-substance granules. This is due to the fact that the lipid granules are so closely packed in the basal region of the cell that it is not possible to make out any underlying structure. But if the Sudan black of such preparations is extracted and the slides restained with iron haematoxylin, one can clearly make out fibrillar mitochondria in the region of the cell where the densely packed sudanophil material was lying. Most of them have a deeply stained granule at each end. One can also see a few deeply-staining granules lying separately amongst the mitochondrial fibres, whereas some of the mitochondrial fibres are devoid of the tip-granules. It appears to the author that these granules, which seem to correspond to the *Praesubstanz* of Hirsch (1939) and the pre-substance of the author (Rishi, 1956), arise as tip-granules of the mitochondria, from which they later disassociate. These pre-substance granules seem to act as nuclei for the lipid synthesis, and later become 'Golgi' granules (lipid granules) by the accumulation of lipid round them. Such a view has also been advocated by Bourne (1951).

It is interesting that the mitochondrial origin of lipid granules and the subsequent conversion of the latter directly into secretory granules has been described by Fujimura (1921) in the human placenta and decidua.

In none of the fixed preparations has the author been able to make out any

structure that could be homologized with the 'canaliculi' described by Lacy (1954) in the cells of the pancreas.

In conclusion it may be stated that in the liver-cells of *Anadenus* there are structures that can possibly be compared with the networks of Golgi.

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The Supposed Pre-pupa in Cyclorrhaphous Diptera

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Summary

The pre-pupal cuticle generally described for Diptera Cyclorrhapha is here con-
sidered to be an inner layer of larval endocuticle rather than the cuticle of a distinct
pre-pupal instar. Chemical and microscopical evidence is given. The mechanism of
rupture of the operculum is also discussed in connexion with endocuticle secretion,
sclerotization, and subsequent water loss.

Introduction

THE secretion of a pre-pupal cuticle before pupation would appear, from
the literature, to be general for the Diptera Cyclorrhapha. It is referred
to in all the current textbooks of insect morphology and physiology, including
Wigglesworth (1950) and Roeder (1953). The instar has also been used as
a subject for physiological investigations, including that on *Drosophila* by
Ellenby (1953). It is mentioned specifically for *Rhagoletis* by Snodgrass (1924),
for *Calliphora* by Tate (1953) and Wolfe (1954*a*, *b*, 1955), for *Sarcophaga* by
Dennell (1946, 1947), for *Drosophila* by Robertson (1936) and Bodenstein
(1950), for *Psila* by Ashby and Wright (1946), and by Fraenkel (1938) for
Calliphora, Lucilia, Phormia, and *Sarcophaga*.

There is amongst the large literature on the pre-pupal cuticle no consistent
account of its macroscopic structure. Snodgrass (1924), who appears to have
been the originator of the term 'pre-pupal cuticle', describes a distinct pre-
pupal stage in *Rhagoletis*, which he considers to represent a reduced fourth
larval instar. According to him the pre-pupa, enveloped in its cuticle, can be
removed from the puparium at a stage between the beginning of puparium
formation and the formation of the true pupa. On the other hand, Fraenkel
(1938) describes the pre-pupal cuticle in *Calliphora* in the abdominal region
only. Robertson (1936) in *Drosophila* describes the pre-pupal cuticle as com-
pletely enveloping and extending into the larval stomodaeum and proctodaeum.
Ashby and Wright (1946) are obviously puzzled over the spatial relationships
of the cuticle in *Psila* and are more critical than other authors of its relation-
ships. Hinton (1946) in discussing the pre-pupal cuticle in Diptera Cyclor-
rhapha accepts Snodgrass's (1924) conception of a reduced fourth larval
instar. Hinton's diagrammatic representation (see fig. 4, A, p. 248) is, more-
over, reproduced in Brauns (1954).

Dennell (1946, 1947), in detailed work on the cuticle of *Sarcophaga*, simply
refers to the fact that Fraenkel and Rudal (1940) noted a pre-pupal moult in
Calliphora, and mentions a pre-pupal moult in *Sarcophaga* in vague, uncon-
vinced terms. Similarly, Wolfe's (1954, 1955) detailed account of the origin

and fate of the third larval (1954*a*) and of the pupal and adult cuticles (1954*b*)
in *Calliphora*, makes only passing mention of the pre-pupal cuticle (1955).
In spite of the large literature on the deposition, sclerotization, and moulting
of the cuticles of the various instars in different species of Cyclorrhapha, no
account exists of the chemical composition or cuticular nature of this very
fine membrane, except the brief mention by Wolfe (1955).

<div style="text-align:center">RESULTS</div>

<div style="text-align:center">Drosophila melanogaster Meigen</div>

The cuticle was first encountered by the author during an investigation into
the development of the tracheal system in *Drosophila melanogaster* (Whitten,
1957). In this small species a pre-pupal cuticle was isolated with difficulty and
it appeared to lie free in the abdominal region, in a similar manner to that
described by Fraenkel (1938) for *Calliphora*, but anteriorly it appeared, in
Drosophila, to be continuous with the inner surface of the operculum. As the
tracheal lining is continuous with the general cuticle of the body surface,
detailed study of the former necessarily involved consideration of the cuticles
in the different instars. I became increasingly aware of the anomalies presented
by this cuticle, particularly with respect to the tracheal system: although
distinct functional tracheal systems are present in each of the larval, pupal, and
adult instars, there is no evidence for a pre-pupal tracheal system. Since the
tracheal system of *Calliphora erythrocephala* Meigen was also under con-
sideration, the latter species, because of its larger size, has been used for
further investigation into the extent and possible nature of the pre-pupal
cuticle. The following represents the results of this work, and the conclusions
which have been drawn from it and from a simultaneous study of *Phormia
terrae-novae* Robineau-Desvoidy.

<div style="text-align:center">Calliphora erythrocephala and Phormia terrae-novae</div>

Puparium formation and the spacial relationships of the cuticular layers

As in all Diptera Cyclorrhapha, puparium formation begins with cessation
of feeding, eversion of anterior spiracles, and darkening of the cuticle.

Darkening is a gradual process and takes place progressively inwards. In
the present instance it has been found possible to peel away the outer sclero-
tized from the unsclerotized inner layers. Whilst darkening is still in progress
it is not possible to remove the operculum; this is quite clearly due to factors
involved in the sclerotization and subsequent loss of water in the 'line of
fracture', and will be discussed later. As darkening continues the body con-
tents at the posterior end are withdrawn from the anal papillae (fig. 1, A);
withdrawal at the anterior end has not yet occurred and the larval tracheae
are still intact.

Later, the mouth armature and living contents anteriorly are drawn in-
wards, and the pupal spiracles make their appearance median to the larval
spiracular tracheae. At this stage the space between the larval cuticle and the
developing pupal spiracles is filled with fluid. Slightly later the intima of the

piracles can be seen secreted simultaneously with that surrounding the larval
runks anteriorly (fig. 1, B).
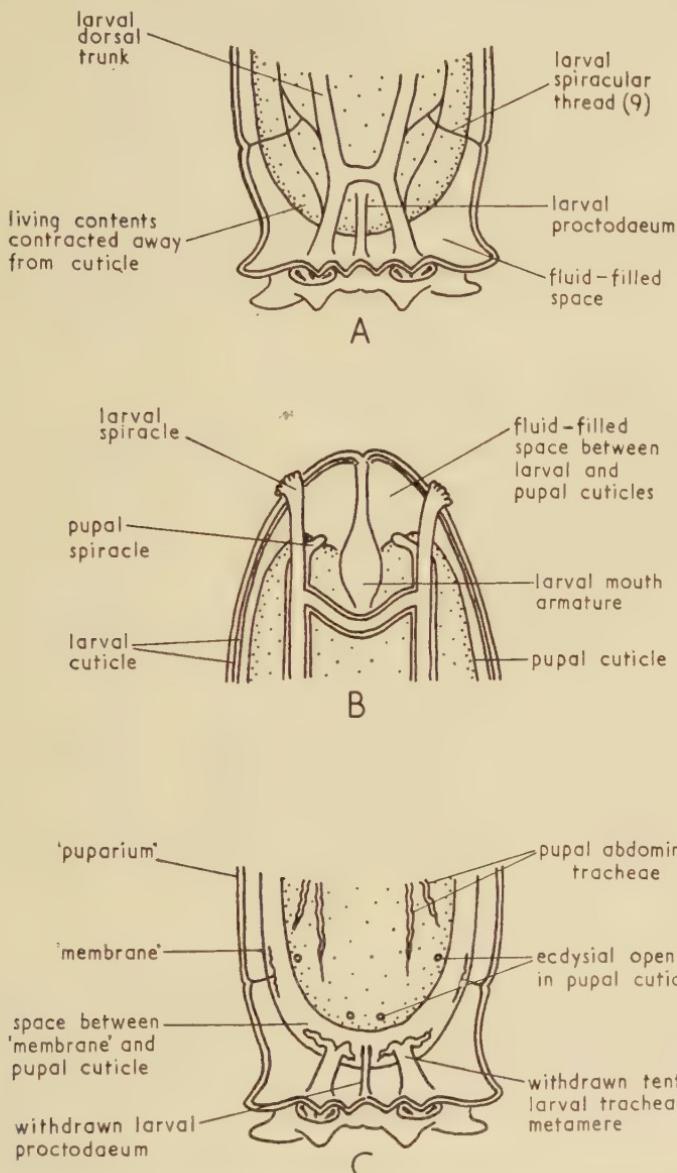
FIG. 1. *Phormia terrae-novae*. A, posterior end of early puparium showing contraction of living
:ontents away from cuticle (compare fig. 3, B). Semi-diagrammatic. B, anterior end of slightly
ater stage showing spatial relationships including retracted larval mouth armature, and re-
racted larval spiracular tracheae in relation to newly formed pupa (compare fig. 3, D). Semi-
liagrammatic. C, posterior end of fully hardened air-filled puparium showing spatial relation-
hips of the pupal cuticle, the 'membrane' or so-called 'pre-pupal' cuticle, and the puparium
vall; larval tracheae and proctodaeum withdrawn (compare fig. 3, E). Semi-diagrammatic.

Dissection of the contents of the puparium at this stage reveals an object intermediate in appearance between larva and pupa. Anteriorly, as already mentioned, the pupal spiracles are visible; the wings and legs are only partially, at most, evaginated, and the head is invaginated. The larval mouth armature and stomodaeum are still retained within the body, and can either be withdrawn from the invagination lying between the pupal spiracles, or left within this by severing their connexion with the puparium anteriorly. The rest of the body consists of the long abdomen, only slightly shorter than the corresponding larval abdomen. The larval tracheae, in both abdomen and thorax, are still retained and the spiracular tracheae connect with the spiracles on the puparium. Successively older puparia, when dissected, reveal specimens intermediate between this and the normal pupa. Quite evidently evagination of the head and thoracic appendages and relative increase in length of the thoracic region is accompanied by progressive contraction of the enveloping cuticle in the abdominal region. Complete contraction of the abdomen to characteristic pupal form is accompanied by withdrawal of the larval tracheae, which also seems to be brought about in part by the release of the air-bubble, hitherto contained within the abdominal region. Tate (1953) describes in detail the part played by the air-bubble in removal of the tracheae, although he offers no explanation of its origin. Bodenstein (1950) illustrates a pre-pupa of *Drosophila* which is clearly comparable with this early pupal stage in which appendages and head are still inverted, larval tracheae retained, and abdomen long and larva-like. It is clearly not a pre-pupa as the inner surface of the puparium at this stage always has the membrane corresponding to the 'pre-pupal cuticle' of other authors lying on its inner surface. Also, the spiracular tracheae which pass through the membrane have either to be severed or artificially withdrawn from the young pupa before it can be removed from the puparium. At all stages the membrane is loose posteriorly and closely applied to the wall of the puparium anteriorly. In older, air-filled puparia, air frequently separates the membrane from the puparial wall. The cast larval tracheae always lie on its inner surface.

Endocuticle secretion and sclerotization in specialized areas

In certain areas of the puparium the endocuticle evidently remains unsclerotized. This is the case with the pupal 'windows' (fig. 2, A) which remain transparent and unsclerotized. Wolfe (1954) states that in *Calliphora* none of the endocuticle remains unsclerotized, but he is specifically concerned with the abdominal region, and in this region the so-called pre-pupal cuticle lies loosely and is unconnected with the puparium wall. The significance of this will be discussed later.

Another region of the puparium in which both secretion and sclerotization of endocuticle is specialized and differs from the general surface is along the lines of rupture of the operculum. In partially sclerotized puparia a lighter band can be seen running down from the pro-thoracic spiracles on each side. When darkening is complete, together with loss of water by the larval cuticle

nd replacement of the fluid internally by air, the region appears as an air-
lled line (fig. 2, A); and the split now occurs with application of only light
ressure. Ease of removal would appear to be dependent on drying out of the
uparial cuticle. Before this, more effort is required to peel away the opercu-

ɢ. 2. *Phormia terrae-novae.* A, anterior end of air-filled puparium in lateral view showing
ture line of fracture of operculum. B, transverse section of larval cuticle in region of line of
acture: early stage of sclerotization and puparium formation. C, anterior end of late air-
led puparium with removed operculum. D, enlarged view of section of torn edge along line
of fracture of operculum. A-D all semi-diagrammatic.
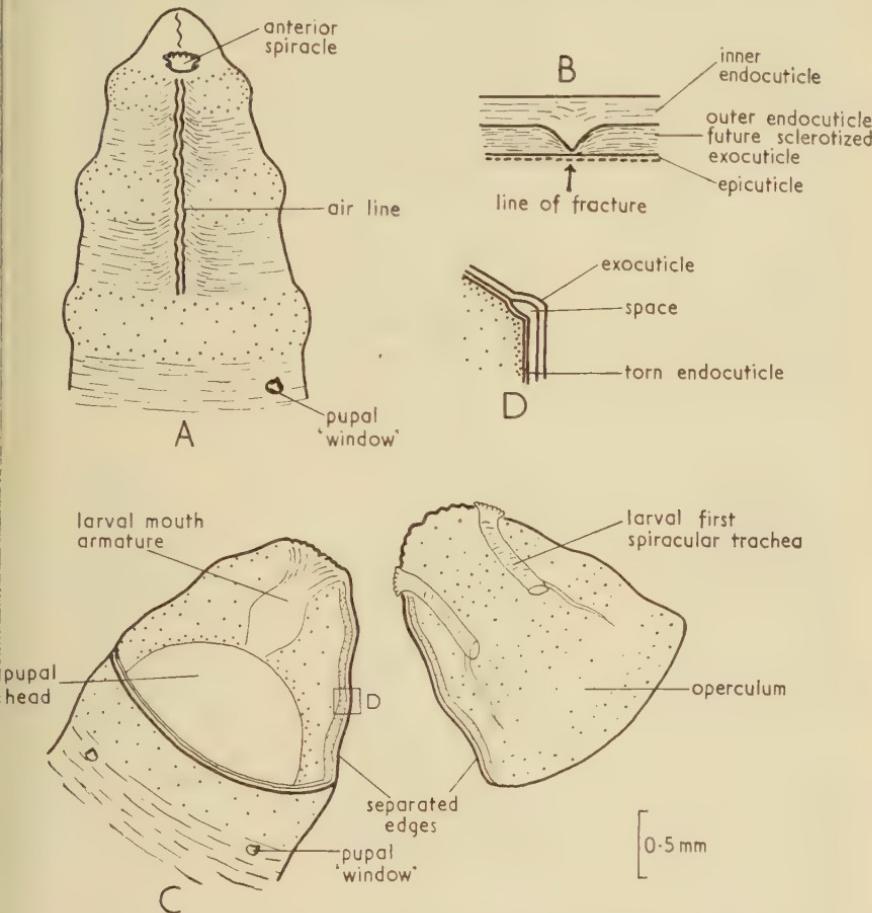
m. The explanation can be found in a transverse section of this region.
rozen sections before hardening and loss of water appear as in fig. 2, B. The
lerotization of the exocuticle is uneven, or rather the actual secretion of
ndocuticle which will become sclerotized (future exocuticle) would appear to
e uneven. In consequence, the underlying region also appears to be secreted
nevenly. A later stage, when drying out has occurred and the operculum is
sily removed, reveals the separated edges of the operculum and antero-
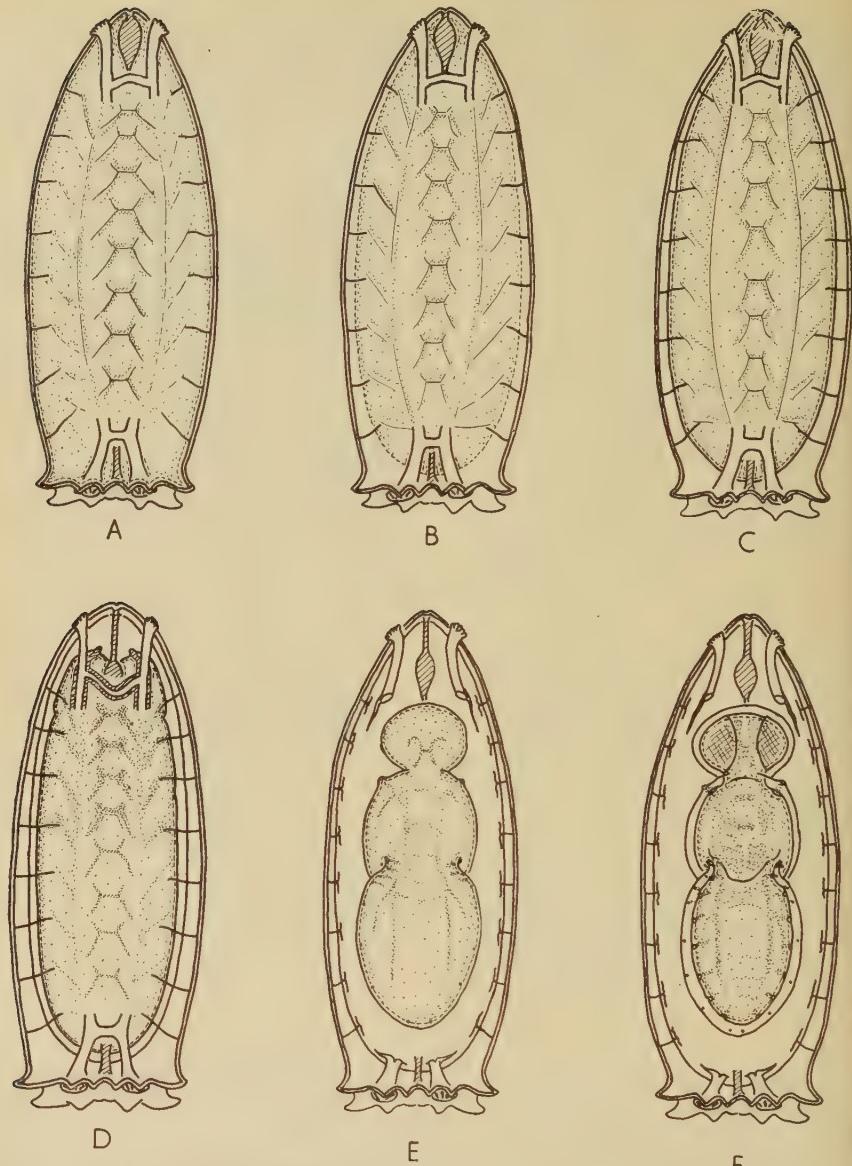
FIG. 3. Diptera Cyclorrhapha: diagrammatic reconstruction of the spatial relationships of the cuticular layers and the hypodermis (in dotted line) at the various stages between the beginning of puparium formation and secretion of the adult cuticle. A, third instar larva in immobile condition at white puparium stage. B, third instar larva with anterior spiracles as yet unretracted but with living contents withdrawn from anal papillae; darkening of puparium beginning. C, third instar larva, darkening proceeding, showing relative positions of outer endocuticle (forming sclerotized exocuticle) and newly secreted inner endocuticle (later forming the membrane formerly considered to be the pre-pupal cuticle). D, early pupa with inverted head and thoracic appendages; larval mouth armature, stomodaeum, proctodaeum, and tracheal system still retained within newly secreted pupal systems; darkening of puparium almost complete. E, late pupa with head and thoracic appendages everted; puparium air-filled and fully sclerotized with larval stomodaeum, proctodaeum, and tracheal system withdrawn; cuticular layers dehydrated and reduced in thickness; exocuticle hard, dark, and brittle; endocuticle transparent and membrane-like. F, adult stage within pupal cuticle, with newly secreted adult tracheal linings surrounding pupal tracheal linings.

ntral surface of the puparium as in fig. 2, C, D. The hard, darkened exocuticle
the separated edge is extremely thin, and is separated by a space from a thin
embrane also apparently torn along its edge. The elongated air-space seen
surface view is presumably the area between the two layers. Except along
is line the inner layer lies close to the hard sclerotized layer. Loss of water
nd resulting decrease in thickness of the two layers in this region would
ppear to cause the separation, the resulting space becoming the elongated
r-space after entry of air into the puparium. The membrane is undoubtedly
e inner endocuticle reduced in thickness by loss of water, separated at the line
f fracture but elsewhere in contact with the sclerotized exocuticle.

hemical nature of the cuticle

Chemical tests applied to this membrane (so-called pre-pupal cuticle)
moved from the abdominal region where it is loose, have given the following
sults: it is stained pale blue with Mallory, whereas the outer exocuticle
ains orange; it stains very faintly with methylene blue; gives a positive test
ith aniline blue, but a negative one with acid fuchsin. The same results were
ven by the withdrawn larval proctodaeum and peeled-off inner layers of the
uparium before sclerotization of the larval cuticle. These results differed
om those given by the pupal cuticle after emergence of the adult, in which the
docuticle has been dissolved leaving epicuticle only (Wolfe, 1954).

Finally, it is soluble in concentrated sulphuric acid, and gives a very positive
action for chitin with the chitosan test.

Discussion

The evidence so far obtained substantiates the hypothesis that this mem-
ane in *Phormia* and *Calliphora* is in fact modified larval endocuticle and not
e cuticle of a distinct pre-pupal instar as is generally supposed.

According to Wolfe (1954), endocuticle is secreted throughout the third
rval instar and is converted, at puparium formation, into sclerotized exocu-
cle, but he considers that no endocuticle remains unsclerotized. However,
olfe is only concerned with the abdominal region and here the 'pre-pupal
ticle' (inner endocuticle) is loose and separate from the exocuticle (sclero-
zed outer endocuticle). Thus any relationship with the larval cuticle is not
vious. Frozen sections of early puparia show the two distinct zones of
docuticle; these are most clearly distinguishable in the region of rupture of
e operculum (fig. 2, B).

In *Sarcophaga* Dennell (1946) has shown that endocuticle is secreted not
ly throughout the third larval instar, but also after the beginning of puparium
rmation. The evidence presented here suggests that this is also the case
Phormia and *Calliphora*; it is this endocuticle secreted during puparium
rmation which remains unsclerotized and subsequently, after loss of water,
comes the thin membrane hitherto known as the pre-pupal cuticle. Com-
rison of the inner unsclerotized endocuticle of *Sarcophaga*, as described
Dennell (1946), with this membrane in *Phormia* and *Calliphora* confirms

their homologous nature. The staining reactions are similar and, in both, pore canals are absent. In addition, both are not dissolved by the moulting fluid occupying the space between larval endocuticle and newly secreted pupal epicuticle. In contrast to this, both at the previous ecdyses and when the adult emerges, the moulting fluid dissolves the endocuticle, leaving only epicuticle as the cast skin (Wolfe, 1954).
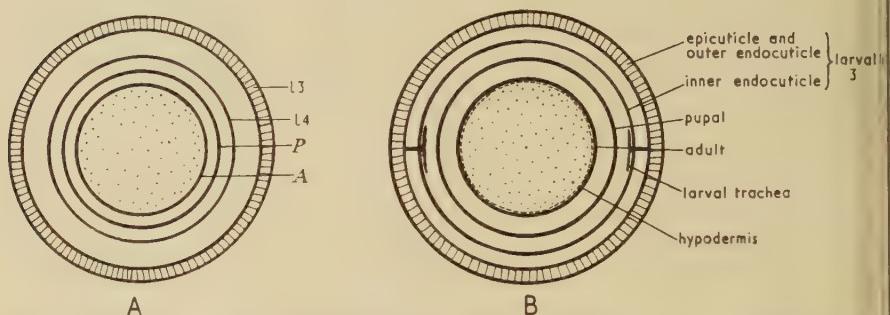

FIG. 4. Transverse section of late puparium in Diptera Cyclorrhapha. Diagrammatic. A, after Hinton (1946, fig. 1). B, reinterpretation according to present results.

The secretion of endocuticle after the beginning of puparium formation can in part explain the spatial relationships of the resulting membrane (inner endocuticle) to the exocuticle (outer endocuticle) of the puparium. Anteriorly the two layers are closely applied although they can be peeled off from one another. This is not surprising since even in the outer endocuticle it is possible to peel off unsclerotized from sclerotized layers during darkening of the puparium, and in the present case the differing chemical and physical composition of the two layers, combined with loss of water by both, probably aids the separation process.

The peculiar separation of the membrane posteriorly could be explained as follows: At the beginning of puparium formation, while the anterior end with its mouth armature has not yet retracted, there is, posteriorly, a withdrawal of the larval tissues from the anal papillae and from the puparial wall (fig. 1, A). If secretion of endocuticle is continuing at the same time, as would appear, then this would result in a separation of the two layers. The separation is subsequently made more obvious after loss of water by both layers and entry of air into the puparium.

The structural relationships seen in *Phormia* and *Calliphora* have been found to be similar in *Drosophila* and *Musca*. As the description of a 'prepupal cuticle' has also been given, by various authors, for many other species, the results given here seem to be general for Diptera Cyclorrhapha. The specialized method of secretion and the subsequent fate of the cuticular layers at the lines of rupture of the operculum, described here, is also probably similar in other Cyclorrhapha; however, the actual extent and positions of the lines vary considerably within the group (Hennig, 1952; Brauns, 1954).

Conclusions

Since the pre-pupal cuticle is in effect the inner larval endocuticle and no pre-pupal instar exists, the pre-pupa of *Rhagoletis* (Snodgrass, 1924) is really still the third larval instar in an immobile condition. The same applies to each of the other examples given in the literature cited. On the other hand, the pre-pupa illustrated by Bodenstein (1950) is really an early pupa. Snodgrass's pre-pupa would correspond to my fig. 3, C and Bodenstein's to my fig. 3 D. Hinton's diagram (1946, fig. 1) would be relabelled as in my fig. 4, B.

Only this interpretation of the membrane in question can resolve the anomalies presented first by the larval tracheal system and the larval stomodaeum and proctodaeum and, secondly, by the inconsistent and unconvincing reports of a distinct instar and its cuticle in the various members of the Cyclorrhapha.

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The Growth of Mixed Populations of *Chilomonas paramecium* and *Tetrahymena patula*

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With one plate (fig. 1)

Summary

In mixed cultures, where *Tetrahymena patula* preys on *Chilomonas paramecium*, the growth of the *Chilomonas* population does not initially differ significantly from that in single cultures. Later, however, the decrease in numbers of *Chilomonas* is more rapid in mixed cultures, where it dies out after two months.

The composition of the polymorphic *Tetrahymena patula* population depends on age and on the presence or absence of *Chilomonas*. Slit-mouthed forms are absent at the beginning of growth in both mixed and single cultures, and only appear subsequently in mixed cultures. The percentage of these forms increases in older cultures. The percentage of microstomatous forms is larger, however, at the beginning of population growth, both in mixed and single cultures. The percentage of macrostomatous forms increases from the beginning, reaches a maximum, and then decreases. Cannibalism has been observed in old mixed cultures.

Significant differences in the size of individual organisms of *Chilomonas* between single and mixed cultures appeared from the beginning of the logarithmic phase of growth, and were pronounced up to the end of observations. Significant differences in the size of *Tetrahymena patula* between single and mixed cultures appeared somewhat later, from the sixth day onwards.

The differences in size and shape between organisms in cultures of the same kind but of different ages were also significant. Variation in size and shape of both species was greater in mixed than in single cultures.

Introduction

EXPERIMENTAL studies of the relations between predator and prey have not been as numerous, nor have they been made on as many different species, as have studies on competition between organisms. Gause (1934, 1935*a*, 1935*b*) showed that their interaction did not invariably lead to periodic oscillations of population density, as predicted in the mathematical theories of Lotka (1925) and Volterra (1926), but that the occurrence of oscillation depended on certain characters of the species under investigation, and on environmental factors. He observed (Gause, 1935*b*) oscillations with *Paramecium bursaria* and *Saccharomyces pombe*, provided that yeast was added to the medium from time to time, and that the population of the predator was decreased by taking samples. In other experiments (Gause, 1935*a*), where *Bursaria truncatella* was predator and *Paramecium bursaria* prey, periodic fluctuations did not appear, however, and *Bursaria* died out before the paramecia were destroyed. Gause does not mention that the relationship was more complex here than in his previous experiments on *Paramecium*

bursaria and *Saccharomyces pombe*; but evidently the food chain of *Bursaria—Paramecium–Saccharomyces* was more complex than the simple relation of predator to prey. According to Sandon (1932), *Bursaria truncatella* feeds on ciliates as well as taking other food. In Gause's experiments *Bursaria* may have preyed both on *Paramecium* and on yeast. According to Lund's observations (1914*a*, 1914*b*), *Bursaria truncatella* is selective in its feeding, since it accepted fragments of hard-boiled yolk stained with dyes insoluble in water, but refused fragments of yolk stained with dyes soluble in water; but Schaeffer (1917), was unable to agree that *Bursaria* had any food-selecting mechanism whatsoever; the occasional refusal of food was only part of a general reaction to more or less injurious stimuli. If *Bursaria* is not selective it is probable that it was indeed feeding on both paramecia and yeast in Gause's experiments.

Gause (1934) reported that *Didinium nasutum*, a voracious predator, destroyed *Paramecium caudatum* and then died out; fluctuations in the densities of their populations did not normally appear. Only when their interaction was modified by the presence of a 'refuge' (a sediment of yeast) or when, as in other experiments, he introduced both species into the culture at regular intervals of time, did fluctuations occur.

Brown (1940) observed the growth of mixed populations of *Leucophrys patula* preying on *Glaucoma pyriformis*. He was interested in the logarithmic phase of the growth of *Leucophrys* and he prepared both theoretical and observed growth curves for that phase, but followed the growth of the cultures for 4 days only, so that his experiments give very limited information about predator-prey relations. In his equations for rates of growth, Brown took the values for *Leucophrys* from the graph representing its growth in single populations, but he did not define the conditions under which it was grown in single populations. At that time *L. patula* (*Tetrahymena patula* according to Corliss, 1952) had not yet been established in bacteria-free cultures. It is doubtful, therefore, whether he was justified in taking values for its growth from graphs based on figures derived from growth under quite different conditions. Nor is it clear why Brown ascribed the end of the exponential phase to the high concentration of predator in the one instance, and to the exhaustion of food in the other. The density of *Leucophrys* was as high in the second instance as in the first, or even higher, as is shown by the curves. He does not state from how many cultures or samples data were obtained.

The study made by Dewey and Kidder (1940) of the growth of *Perispira ovum* with *Euglena gracilis* gives more details and covers a longer period than Brown's data. It is interesting to note that *Perispira* continued multiplying for some time after the extinction of its prey, and at the expense of the size of individuals.

Lilly's study (1942) of the nutrition of *Stylonychia pustulata* and *Pleurotricha lanceolata* is also important as a contribution to knowledge of the growth of mixed populations. It reveals how small details gathered from
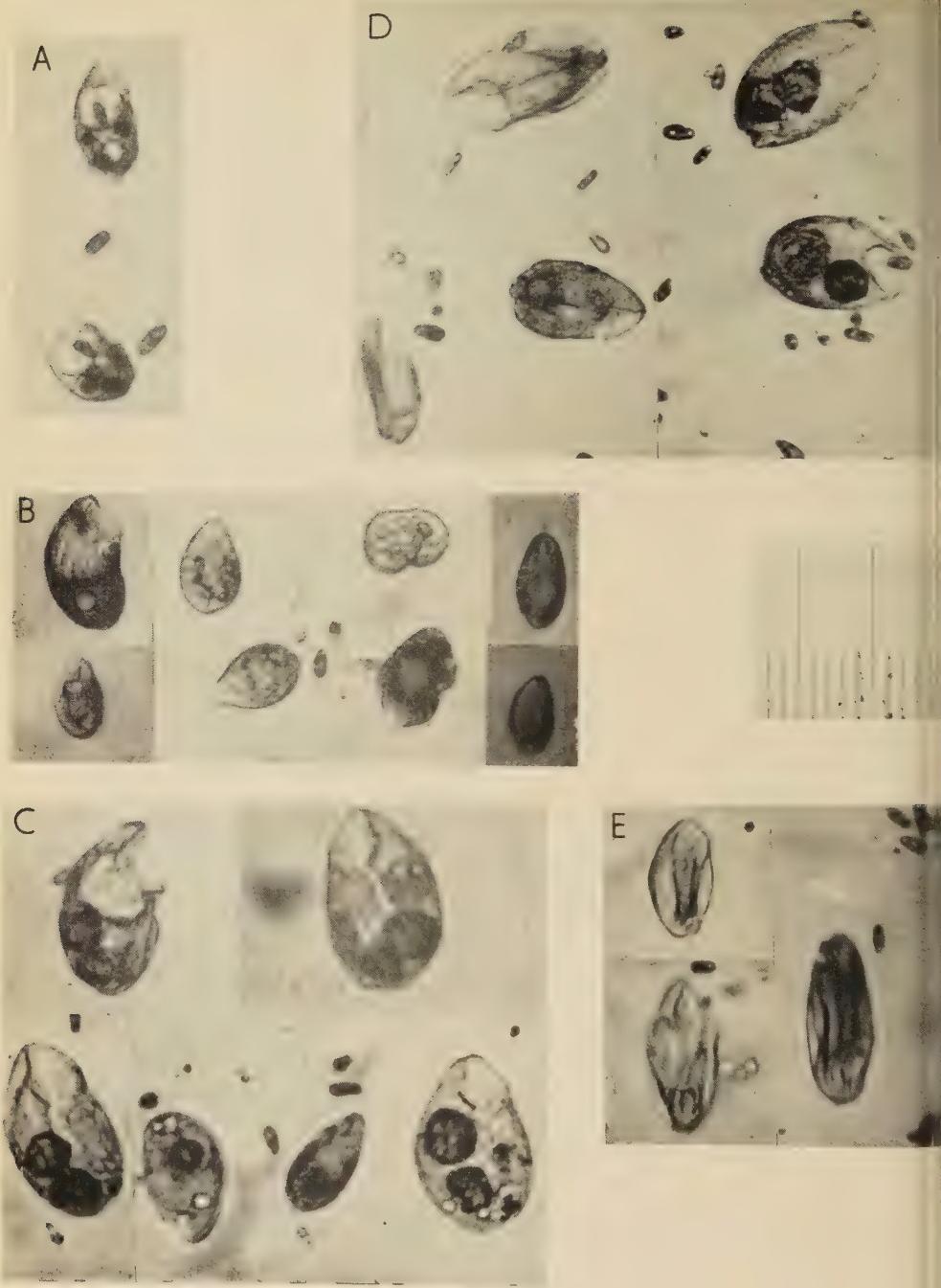
Fig. 1

S. MUČIBABIĆ

observation of the species may be of value in explaining the growth of the population. At the same time, it implies how many variables are necessarily neglected, if the growth of mixed populations—for example, the interaction between predator and prey—is approached purely mathematically. Lilly confirmed that the length of the lag phase for these ciliates depended on the age of the inoculum. He also found that the size of the organisms is important in relation to the duration of the lag phase, which lasted longer if the inoculum consisted of small organisms, because these are not able to ingest large food organisms until they have increased in size. Lilly compared the phases of growth of both predators, and reported that they were characteristic for each species. Differences between the growth of their populations also appeared when the food was exhausted: *Pleurotricha* continued to grow, whereas *Stylonychia* did not.

According to this review of previous work, the growth of mixed populations of two protozoan species which are related as predator to prey, has previously been studied either in small communities composed of three species at least, or on two species in bacteria-free cultures but without control experiments. It seemed desirable, therefore, to examine the predator-prey relationship in a community of two species with control cultures of the single species.

MATERIAL AND METHODS

In preliminary experiments, two species of Protozoa were sought, able to live separately in pure axenic culture in the same organic medium, but which, when brought together, interact as predator and prey. *Chilomonas paramecium* serves as prey to many ciliates, but since most of these are carnivorous forms that cannot live without food organisms, even in an organic medium, choice was restricted to those few ciliates which have been obtained in bacteria-free cultures. Among the flagellates, *Peranema trichophorum* feeds upon *Chilomonas* (Chen, 1950), but it cannot live with *Chilomonas* in the same organic medium. An attempt was first made to use *Tetrahymena vorax* (strain V_2) as predator. Mixed cultures were inoculated with macrostomatous forms only, because these are known to be carnivorous. They fed on *Chilomonas*, but after 3 to 4 days all macrostomatous forms had disappeared, and the population was composed of microstomatous forms only, which are not predaceous (Kidder, Lilly, and Claaff, 1940). This species, therefore, was abandoned. Subsequently, *Tetrahymena patula* (Müller) Corliss was found to be suitable for the present experiments; it takes *Chilomonas* as food (fig. 1, A) and can also

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FIG. 1 (plate). A, *Tetrahymena patula* with ingested *Chilomonas paramecium*, from a culture 5 days old.

  B, macrostomatous, microstomatous, intermediate, and slit-mouthed forms of *Tetrahymena patula*.

  C, cannibalistic forms of *T. patula*.

  D, forms of *T. patula* with re-entrant posterior end.

  E, forms of *T. patula* showing longitudinal folds.

  Each smaller division of the scale represents 10μ.

live as a saprophyte. Subcultures were obtained from the Culture Collection of Algae and Protozoa, Botany School, Cambridge.

Maupas (1888) wrote on *Tetrahymena patula* under the name *Leucophrys patula*, giving a description of the species and many observations on its life-cycle. Fauré-Fremiet (1948) extended Maupas's description, retaining the name *Leucophrys*. Recently, however, Corliss (1952, 1953) gave a full account of the history, systematics, and morphology of this species as *Tetrahymena patula*. *T. patula* was isolated before 1942 by Fauré-Fremiet (Corliss, 1952).

It was in principle desirable to perform experiments with the same medium as had been used in the experiments on *T. pyriformis* (Mučibabić, 1957) but this was not possible because in the medium of 0·1% proteose peptone and 0·1% sodium acetate, *T. patula* did not continue to multiply after one or two initial divisions. The medium became satisfactory, however, when the concentration of proteose peptone was increased from 0·1% to 1%.

Cultures in 1% proteose peptone were inoculated with 10 *Chilomonas* and 10 *Tetrahymena patula*, and control cultures were inoculated with 10 organisms of each species separately. All cultures were maintained at 22·5° C. The number of organisms was counted every day as in previous studies (1956); but since the density of population of *Chilomonas* differed greatly from that of *Tetrahymena patula* from the third day onwards, the size of the two populations could not be determined in the same way. Five samples from each culture were counted in order to determine the size of the *Chilomonas* population; while the entire population of *Tetrahymena patula* in each culture was counted throughout the period of the experiment. As the culture had to be diluted before the population of *Chilomonas* could be counted, *Tetrahymena patula* was counted before dilution. The counting-procedure was as follows.

One drop of culture was placed on a slide. Since during the counting of *T. patula* it was not possible to pick up *T. patula* alone, the organisms collected in the capillary micro-pipette during counting were not discarded, but were delivered into a test-tube containing 5 to 10 ml of fresh medium. The next drop was placed on the same spot on the slide, and so on, till the whole culture had been examined. When the last drop had been taken, 0·3 ml of the medium was pipetted into the culture test-tube. This was also examined, in order to pick up any organisms that might have remained on the wall of the test-tube. The individuals of *Chilomonas* remaining in the drop on the slide were washed from the slide into the large test-tube in which the whole culture had been diluted. For washing, 5 ml of the fresh medium was used. Finally, the necessary quantity of medium was added to complete the required dilution. From this diluted culture 5 samples were then counted, in order to determine the size of the *Chilomonas* population.

Growth of single and mixed populations of Chilomonas paramecium *and* Tetrahymena patula *in terms of total number of organisms*

Population growth of *Ch. paramecium* and *Tetrahymena patula* in terms of

total number of organisms is shown in fig. 2 and table 1 (p. 256). The table shows mean size of the population, standard deviation, and number of observations for both species in single and mixed cultures. Table 1 and fig. 2
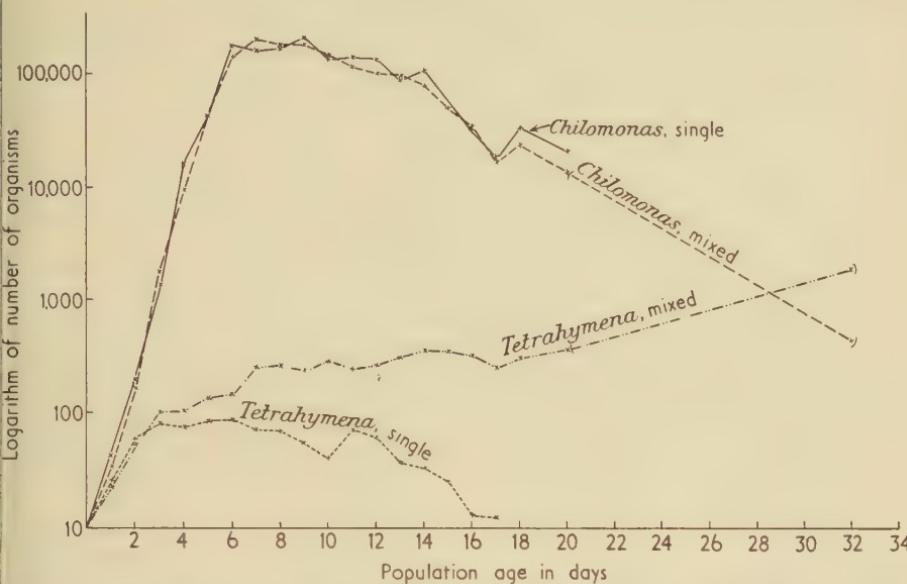
Fig. 2. Graph showing the growth of populations of *Chilomonas paramecium* and *Tetrahymena patula* in single and mixed cultures, in terms of total number of organisms.

reveal that the growth rate of the *Chilomonas* population is very similar in single and mixed cultures. A very pronounced lag-phase appeared in both single and mixed cultures in this medium. It lasted for 2 days. The maximum numbers of *Chilomonas* in single and mixed cultures do not differ significantly.

At the beginning, the growth of the population of *Tetrahymena patula* is very similar in single and mixed cultures. Differences appear from the 3rd day onwards and later become greater. From the 6th day, the population of *Tetrahymena* in single cultures begins to degenerate: the organisms become very opaque, flattened, and abnormal, and their number decreases rapidly. In mixed cultures, however, tetrahymenae were multiplying until the 32nd day. On the 6th day they began to take *Chilomonas* more noticeably than before, and organisms with ingested individuals of *Chilomonas* were frequent (fig. 1, A). The following day the population of *Tetrahymena patula* was nearly twice as great. This outbreak of division of *Tetrahymena* after increased predatory activity on the 6th day was observed in both series of experiments. The population of *Tetrahymena* increased from then onwards with small fluctuation till the end of experiment. Since the single culture of *Tetrahymena* had died out by the 18th day, counting of mixed cultures was not continued after the 20th day, but occasional observations were made from time to time. On the 32nd day the entire population of *Chilomonas* and of *Tetrahymena*

TABLE 1

Growth of single and mixed populations of Chilomonas paramecium *and* Tetrahymena patula

Age in days	Single populations						Mixed populations					
	Chilomonas			Tetrahymena			Chilomonas			Tetrahymena		
	$\bar{x}$	s	n	$\bar{x}$	s	n	$\bar{x}$	s	n	$\bar{x}$	s	n
0	10			10			10			10		
1	42	5·5	4	25·2	6·3	6	34·5	11		23	1·7	6
2	197	49·6	4	60·3	8·0	6	173·5	55		54	15·4	6
3	1,383	375	4	83·3	20·4	6	1,825	642		102·1	28·8	6
4	16,700	5,100	4	78·9	59·5	7	9,710	3,740		105·6	60·7	6
5	44,500	10,400	4	87·7	49·2	6	44,500	19,900		139·3	55·7	6
6	185,000	33,900	4	90·5	14·8	6	147,000	28,000		149	16·1	6
7	170,750	43,400	4	75·6	18·2	6	217,100	27,100		261·3	104·5	6
8	184,750	32,500	4	72·6	12·4	6	190,300	27,200		275·2	139·8	6
9	225,000		1	56·0	19·8	3	194,000	27,000		251·6	48·4	6
10	147,000		1	42		1	156,800	34,800		302·6	203·8	6
11	153,000		1	76		1	125,690	35,000		266·5	50·3	6
12	150,000		1	64		1	110,000	15,200		279·5	92·4	6
13	97,000		1	38		1	104,600	10,500		335·0	94·6	6
14	119,000		1	34		1	88,000	30,447		377·6	173	6
15				26		1	55,000	3,606		373·8	148	5
16	35,000		1	13		1	38,300	9,592		348·3	34·2	3
17	20,000		1	12		1	18,000	5,654		265	69·2	2
18	37,000		1				26,300	1,581		332·0	46·1	3
20	23,000		1				15,000	8,660		389·0	167	3
32							474			1,072		1

was counted in one culture; the population of *Chilomonas* was then very small (only 474 organisms), while that of *Tetrahymena* included over a thousand individuals. After two months the *Tetrahymena* population was still dense, although no *Chilomonas* were to be found, in the originally mixed culture.

Data in table 1 for the *Chilomonas* population in single and mixed cultures were statistically compared, as well as data for the *Tetrahymena* population. On the 4th day only the population of *Chilomonas* is significantly larger in single than in mixed cultures. On the 7th day, however, the population of *Chilomonas* is larger in mixed than in single cultures to a degree that is almost statistically significant.

The difference between the size of *Tetrahymena* populations in single and mixed cultures is significant from the 6th day of population growth, the population being larger in mixed than in single cultures.

Growth of population of Chilomonas *in single and mixed cultures in terms of total volume of organisms*

Table 2 summarizes data for population growth of *Chilomonas* in single and mixed cultures in terms of biomass (total volume of organisms). Total volumes of organisms were calculated from the data in tables 1 (above) and 5 (p. 259) (for method see Mučibabić, 1957). The biomass of *Chilomonas* is

TABLE 2

Growth of population of Chilomonas *in single cultures and in mixed cultures with* Tetrahymena patula *in terms of total volume of organisms (in thousands of cubic* μ)

Age in days	Single population	Mixed population
0	16·9	16·9
2	282	257
4	18,003	12,118
6		175,224
8	193,249	153,001
10	215,776	123,402
14		83,512

tially smaller in mixed than in single cultures. Later, for a short time, it rpasses the amount in single cultures; but as soon as it reaches the maximum ationary phase, the *Chilomonas* population in mixed cultures begins to crease in total volume.

anges in composition of population of Tetrahymena patula *in single and mixed cultures*

Tetrahymena patula is known to be a polymorphic species (fig. 1, B). Forms th a large peristome+mouth (macrostomatous), with a small mouth icrostomatous), and with a small slit instead of an open mouth, have eviously been observed in bacteria-free cultures, as well as in cultures with cteria. Transitional forms between the microstomatous and macrostomatous rms have also been described. In the present work, during the first series experiments, it was noticed that the composition of the *Tetrahymena* pulation changes during population growth. For this reason, in the second ies of experiments the number of each form of *Tetrahymena* present was corded. This prolongs and increases the difficulty of counting, because one s to wait until the organisms are in such a position that the mouth can be en. Occasionally it was not possible to determine by observation in life to ich type an organism belonged. Transitional forms between microstomatous d macrostomatous forms were counted as macrostomatous forms.

The percentage of each form of organism from the whole population was culated, in order to make the results comparable. These are shown in ple 3 (p. 258). The table reveals that slit-mouthed forms never appear in gle populations, while microstomatous forms are more frequent during the arithmic phase of population growth than at other times, both in single d in mixed cultures. Their number in mixed populations increases on the day, at the time when the rate of population growth suddenly increases.

The proportion of macrostomatous forms increases during population wth. Their percentage is greatest when the single cultures are in the

TABLE 3

Composition of population of Tetrahymena *in single and mixed cultures*

Days	Single cultures								Mixed cultures					
	Macrostomatous		Microstomatous		Slit-mouthed forms	Indeterminate			Macrostomatous		Microstomatous		Slit-mouthed forms	Indeterminate
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	$\bar{x}$	n		$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	$\bar{x}$
	%		%			%			%		%		%	%
1	72·54*	17·32	26·13	15·03	..	..	3		27·9	14·3	72·1	14·50	..	..
2	55·51	3·13	44·03	2·57	..	2·46	3		38·74	7·10	41·26	7·41	..	..
3	87·13†	3·57	11·31	5·62	..	1·56	3		68·05	3·24	27·74	1·61	..	4·21
4	86·86	4·61	13·14	4·61	..	..	3		89·81	6·62	9·91	1·71	0·8	..
5	95·19	3·29	4·17	1·44	..	0·64	3		96·64	0·96	2·50	0·31	..	0·86
6	95·42	7·11	1·21		..	3·37	3		95·52	0·24	4·27	0·46	..	0·21
7	83·06	22·0	7·76	6·80	..	9·18	3		64·59	1·48	17·39	3·02	17·68	0·27
8	46·35	10·0	5·88	5·44	..	47·77	3		88·01†	3·56	5·41	1·35	6·58	..
9									79·35		11·03		9·62	..
10									77·14		10·08		12·78	..
11									71·66		8·15		20·20	..
12									64·68		5·97		29·35	..
13									73·80		3·62		22·57	..
14									75·32		4·57		20·11	..
15									77·06		3·84		19·09	..
20									72·27		3·98		22·33	1·42
32									29·66		2·99		67·35	..

     * significant   † highly significant

stationary phase. At the same time their percentage is also maximal in mixed populations; later their number decreases when the slit-mouthed forms appear in greater number. These macrostomatous forms are known to be cannibalistic (fig. 1, C). They prey on the slit-mouthed forms as well as on *Chilomonas*, so that the percentage of both macrostomatous and slit-mouthed forms oscillates. In cultures a month and two months old, slit-mouthed forms are the most numerous; the percentage of macrostomatous forms is smaller than before, but they are large and healthy; microstomatous forms are poorly represented.

This division of forms of *Tetrahymena patula* into three groups is only approximately correct; it does not reflect the variety of shapes in which macrostomatous forms may appear in the same culture. Besides the forms that are rounded at the posterior end, as mentioned by previous authors, there are also forms with a re-entrant posterior end (fig. 1, D), forms with longitudinal folds (fig. 1, E), and forms with an obtusely pointed end. The last were mentioned by Maupas (1888). In cultures 8 days old or more, macrostomatous forms were observed in a striking and characteristic attitude, as if looking for food: the body tilted with the mouth downwards, apparently examining the substratum, while the organism slowly advanced.

On the whole, there are no statistically significant differences between the percentages of microstomatous or macrostomatous forms present in single and mixed populations. The most important difference is the occurrence of slit-mouthed forms in mixed populations only.

Changes in size and shape of Chilomonas paramecium *and* Tetrahymena
patula *during the growth of their single and mixed populations*

Tables 4 and 5 contain data on changes in size and shape of *Chilomonas
paramecium* and *Tetrahymena patula* during the growth of their single and
mixed populations. These data were obtained in the same way as in previous

<div align="center">TABLE 4</div>

Changes in size and shape of Chilomonas paramecium *during populsyion
growth in single and mixed cultures*

<div align="center">c.v. = coefficient of variation</div>

	Single population								Mixed population							
	L		W		L/W		V		L		W		L/W		V	
Days	$\bar{x}$	c.v.	$\bar{x}$	c.v.	$\bar{x}$	c.v.	$\bar{x}$	n	$\bar{x}$	c.v.	$\bar{x}$	c.v.	$\bar{x}$	c.v.	$\bar{x}$	n
0	27·2	12·79	10·9	12·94	2·51	14·54	1,690	16	27·2	12·79	10·9	12·94	2·51	14·54	1,690	16
2	23·5	12·13	10·8	8·86	2·18	10·96	1,434	25	24·3	11·56	10·8	6·71	2·28	13·82	1,482	33
4	21·9	12·44	9·7	17·42	2·20	11·01	1,078	43	23·4	12·56	10·1	10·30	2·32	12·63	1,248	26
6									22·8	12·72	10·0	9·15	2·28	10·09	1,192	37
8	21·7	7·05	9·6	5·80	2·27	8·77	1,046	30	19·9	6·70	8·8	3·45	2·28	10·75	804	40
10	21·2	9·72	9·3	9·0	2·29	8·17	959	21	19·0	10·0	8·9	10·0	2·15	11·49	767	30
14									20·1	14·57	9·5	2·72	2·11	10·14	949	28

<div align="center">TABLE 5</div>

Changes in size and shape of Tetrahymena patula *during population
growth in single and mixed cultures*

<div align="center">c.v. = coefficient of variation</div>

		Single population							Mixed population						
		L		W		L/W			L		W		L/W		
	Days	$\bar{x}$	c.v.	$\bar{x}$	c.v.	$\bar{x}$	c.v.	n	$\bar{x}$	c.v.	$\bar{x}$	c.v.	$\bar{x}$	c.v.	n
macro	0	90·4	11·81	50·6	10·69	1·79	8·16	5	90·4	11·81	50·6	10·69	1·79	8·16	5
micro		63·0	7·74	34·9	11·66	1·83	4·04	10	63·0	7·74	34·9	11·66	1·83	4·04	10
macro	2	84·95	9·98	53·2	11·54	1·60	7·13	21	79·9	12·93	49·0	10·65	1·63	9·14	28
micro		63·0	12·78	37·25	12·79	1·71	13·45	8	64·8	5·77	41·8	11·51	1·57	10·64	10
macro	4	78·5	11·59	49·8	11·49	1·58	7·59	20	79·5	13·66	49·9	19·34	1·71	9·47	17
micro		59·75	1·67	39·0	9·59	1·54	8·90	4							
macro	6	72·9	15·49	40·6	16·50	1·81	12·38	14	81·8	16·28	44·5	12·85	1·85	15·66	32
macro	8	78·7	9·35	44·3	8·01	1·78	7·81	6	105·5	14·79	56·3	16·31	1·89	10·74	27
micro									59·0		39·0		1·51		1
macro	10								95·2	18·70	53·3	15·61	1·81	20·17	18
macro	13								102·0	20·37	53·0	30·35	1·98	19·49	3
macro	14								111·4	21·29	63·6	22·47	1·67	11·14	19
micro									78·0	5·44	40·0	0	1·95	5·44	2
silt-mouthed									54·5	6·09	30·0	2·72	1·82	7·64	4

experiments on the growth of mixed populations of *Chilomonas paramecium*
and *Tetrahymena pyriformis* (1957). The volume of individual organisms of
T. patula, however, cannot be calculated from the data obtained from the
photographs, because of the irregular shape of the animal, and for this reason
data for the volume of *T. patula* do not appear in table 5.

Length, width, and volume of individual organisms of *Chilomonas*, both in
single and mixed cultures, decrease during population growth. The size of

individual organisms increases only when the numbers of the *Chilomon*
population in mixed cultures begin to decrease.

The ratio of length to width of *Chilomonas* shows a decrease after inocu-
tion, in single and mixed cultures, during the lag phase. This means that t
organisms become more plump. In other phases of population grow
changes in shape of *Chilomonas* are not great. In mixed cultures only is the
a pronounced decrease in the ratio at the end of the stationary phase, when t
population of *Chilomonas* starts decreasing.

The length of *Tetrahymena patula* decreases, both in single and mix
cultures, after inoculation. Later, the length increases slightly in single cu
tures, though the organisms never regain the length of freshly inoculat
organisms. In mixed cultures, however, an increase in length is very mark
on the day following the increased ingestion of *Chilomonas*. A further increa
in length was recorded when the organisms become cannibalistic; at t
beginning of population growth they are *not* cannibalistic. The width
organisms also increases in mixed cultures at the same time.

The ratio of length to width of *Tetrahymena patula* decreases after inocu-
lation, and the organisms are plump at the beginning of the logarithm
phase. Later, however, they become slender, even more slender than fresh
inoculated organisms. Data for microstomatous forms are scarce, but suc
as they are, they are shown in table 5 (p. 259) under the data for macro
stomatous forms. The lowest row (on the 14th day) comprises data for th
slit-mouthed forms.

The variation in shape of *Chilomonas* is greater in mixed than in singl
cultures, as can be seen from the coefficients of variation of the ratio length
width (coefficient of variation = relative standard deviation, that is, th
standard deviation expressed as a percentage of the mean). The variation i
size and shape of *Tetrahymena patula* is very great in mixed cultures. At th
beginning of growth, variation is smaller than later.

Using the *t*-test (Fisher, 1950), the data from tables 4 and 5 have been
statistically compared, in order to determine the significance of difference o
means between single and mixed cultures. Significant differences in length
between *Chilomonas* from single and mixed cultures exist from the beginning
of the logarithmic phase of growth till the end of observations. Differences in
width of organisms from single and mixed populations, as well as difference
in the ratio of length to width, are usually not significant.

The comparison of results for *Tetrahymena patula* shows that differences
in width of organisms from single and mixed cultures are always significan
or nearly significant. Differences in length of *Tetrahymena* are significan
from the 6th day, but differences in the ratio of length to width are les
significant than those for length or width.

The mean maximal and minimal values for length, width, and ratio of
length to width, for members of the single populations were compared. This
comparison was also made for members of the mixed populations. Values of
t for *Chilomonas* for the difference between mean maximal value at 2 days and

minimal value at 10 days, from single populations, were: 3·167 for length; 5·681 for width; and 1·756 for the ratio of length to width. Corresponding values for *Chilomonas* from mixed cultures were: for length (2 and 10 days old), 13·022; for width (2 and 8 days old), 7·547; for the ratio length : width (L : W) (6 and 14 days old), 2·737. These values show that the differences are highly significant, with the exception of the difference in the ratio of length to width in organisms in single population which, however, is nearly significant; that is to say, the organisms in single culture change significantly in *size* during population growth, while those in mixed culture change significantly both in *size* and in *shape*. The corresponding values of *t* for *Tetrahymena patula* in single population were: for length (2 and 6 days old), 3·40; for width (2 and 6 days old), 5·70; for the ratio L : W (4 and 6 days old), 3·506. In mixed populations the values were: for length (4 and 14 days old), 5·276; for width (6 and 14 days old), 5·568; for the ratio L : W (2 and 8 days old), 5·416. These are all highly significant; so that both size and shape of *Tetrahymena patula* change significantly during population growth, both in single and in mixed cultures.

DISCUSSION

According to the mathematical theories of Lotka (1925) and Volterra (1926), the interaction between predator and prey is characterized by cyclical variation in numbers of both species. In the present experiments, however, such periodic oscillations in population numbers have not appeared. After reaching the stationary phase, the population of the prey decreased continuously until it was exterminated, while the population of the predator increased steadily. At the beginning of population growth, the size of the *Chilomonas* population in single and mixed cultures does not differ significantly; for a short time, however, the population of *Chilomonas* is greater in mixed than in single cultures. This is, surprisingly enough, at the time when *Tetrahymena* ingests *Chilomonas* more readily than it did before. This must mean that the multiplication rate of *Chilomonas* in mixed cultures is greater at that time than it is in single cultures.

It has been shown that the composition of the population of *Tetrahymena patula* is affected by the presence or absence of *Chilomonas;* it also changes with the age of population. Slit-mouthed forms do not appear in single cultures, while in mixed cultures their percentage increases in old cultures. Corliss (1953) shows slit-mouthed forms as a transitory stage between microstomatous and macrostomatous forms; but in the present experiments, slit-mouthed forms did not appear in single cultures, though both macrostomatous and microstomatous forms were present. It was observed that the slit-mouthed forms swim quickly, and Maupas (1888) also recorded a greater rate of swimming in slit-mouthed forms. He found them in cultures with a small quantity of food. In the present experiments also, the slit-mouthed forms do not appear from the beginning, but only from the seventh day. In one culture only, a single slit-mouthed form was noticed on the 4th day.

Maupas ascribed to them the role of the distribution of species. Fauré-Fremiet (1948) observed slit-mouthed forms in cultures of *Tetrahymena* ('*Leucophrys*') *patula*, previously fed with *Colpidium*. They appeared only in starving cultures. He observed a progressive reduction in the size of the mouth of microstomatous forms; indeed, in some forms the mouth became vestigial, and such organisms were unable to ingest bacteria. He mentions that these forms became the prey of the macrostomatous forms; this was also observed in the present experiments. Fauré-Fremiet and Mugard (1949) have described macrostomatous and microstomatous forms of *Espejoia mucicola*. The microstomatous forms are migratory and were observed to transform themselves to macrostomatous forms as soon as they entered the mucilage covering the thallus of *Batrachospermum*. In *Espejoia* the occurrence of microstomatous and macrostomatous forms does not depend on the age of population, as observed in the present experiments on *Tetrahymena patula*, but on the presence or absence of the appropriate mucilage.

It has been observed that *T. patula* does not prey continuously on *Chilomonas*. After a day of voracious feeding, they seem to 'rest', since on the following day *Tetrahymena* could not be seen to take *Chilomonas*. Volterra and Lotka's equations for the interaction of organisms are based on the supposition that the rate of feeding is constant, and that the finding of prey by predator is a matter of chance. In old cultures, with 'clouds' of degenerating *Chilomonas*, *Tetrahymena patula* was observed to gather in these clouds. It is questionable whether either this aggregation or the discontinuity in feeding is the result of chance.

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